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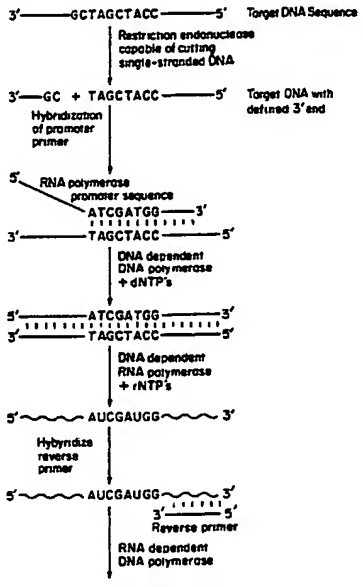

(57) Abstract

An isothermal method for amplifying DNA of interest having a defined 3' end and contained in a target DNA sequence is disclosed. The method comprises combining, under appropriate conditions: the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences; a promoter primer; a reverse primer; at least one RNA-dependent DNA polymerase; at least one DNA-dependent DNA polymerase; at least one DNA-dependent RNA polymerase; an agent with RNase H activity; and appropriate nucleoside triphosphates. In addition, several embodiments of the amplification process are disclosed. For example, a restriction enzyme which recognizes and specifically cleaves either single-stranded DNA or double-stranded DNA at a selected site is also combined with the target DNA, to cleave the target DNA at a selected site, to produce DNA of interest having a defined 3' end.

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IN VITRO, ISOTHERMAL NUCLEIC ACID AMPLIFICATION

DESCRIPTION

Background of the Invention

Several processes have been developed to detect, identify and subsequently isolate a specific nucleic acid sequence within a larger nucleic acid molecule present in small quantities in a sample. One method, the polymerase chain reaction, or PCR, utilizes *in vitro* replication of specific segments of a target nucleic acid of interest. The method is designed to enable the rapid and specific *in vitro* amplification of target nucleic acid sequence using the reiterative, reciprocal interactions of two synthetic primer oligonucleotides with their respective templates and an inducing agent of template-directed synthesis. This method, described in U.S. Patent 4,683,202, is used in a variety of medical DNA diagnostic applications. Numerous PCR variations have been extensively used in biological research and development for the molecular manipulation of nucleic acid sequences.

Enhancements of the PCR method have been reported, including the use of a thermal-stable DNA polymerase from *Thermus aquaticus* which enabled the relatively simple automation of the process by means of a microprocessor controlled thermal cycler. (Saki, R.K., *et al.*, *Science*, *239*: 487-491 (1988)).

In addition, several transcription-based nucleic acid amplification methods have been described. In this process, a double-stranded nucleic acid is produced containing a sequence corresponding to a

target sequence operably linked to a promoter. This double-stranded nucleic acid is subsequently used as a template to produce a plurality of RNA transcripts. (PCT Patent Application, WO88/10315, December 29, 1988; and PCT Patent Application, WO89/01050, February 9, 1989).

PCR and the transcription-based amplification method are time-consuming and labor-intensive processes. The PCR method is tedious because thermal cycling, by hand or by machine, is necessary. The transcription-based amplification method requires that additional reagents be added periodically due to the inactivation of necessary enzymes by the temperatures employed. A cost effective, isothermal method of amplifying a specific nucleic acid sequence would be of great benefit.

Summary of the Invention

The present invention pertains to an isothermal method for amplifying DNA of interest which has a defined 3' end or RNA of interest. The DNA of interest or RNA of interest can be present in a mixture of sequences or purified and can be a portion of a longer sequence, referred to as target DNA and target RNA, respectively. In addition, several variations of the method of the present invention are disclosed for processing target DNA sequence in which a DNA sequence to be amplified (DNA of interest) is present, to produce DNA of interest having a defined 3' end available for hybridization with complementary nucleic acid sequences and subsequently amplifying the DNA of interest. According to the present method, a target DNA sequence containing DNA of interest which

has a defined 3' end is combined with at least two selected primers and a number of selected enzymes, to produce an overall cyclic, isothermal reaction which results in amplification of the DNA of interest.

The present invention provides a "one-pot", isothermal amplification method for amplifying DNA of interest which has a defined 3' end and is contained in a target DNA sequence. Using this method, the specific amplification of DNA of interest can be accomplished at a wide variety of temperatures (i.e., any temperature consistent with the stability of the required enzymes and integrity of the various nucleic acid sequences involved). This is accomplished by employing a number of selected enzymes including, for example, ribonuclease H (RNase H), which functions to degrade RNA hybridized to a DNA sequence. With the addition of an agent or enzyme, such as RNase H which specifically catalyzes the hydrolysis of RNA present in a RNA/DNA heteroduplex, there is no need for thermal cycling to denature intermediates. Thus, the present invention provides an isothermal amplification method which avoids the time-consuming, heating and cooling manipulations necessary to denature intermediates. In addition, the present invention provides a "one-pot" amplification reaction whereby a single mixture of amplification reagents is combined with a target DNA sequence in which the DNA of interest is available for hybridization.

The method described herein can be used to amplify and subsequently detect and/or characterize DNA of interest associated with various pathogens, infectious diseases, and genetic disorders. In addition, the amplification method can be utilized to

clone DNA of interest for insertion into a suitable expression vector.

Brief Description of the Drawings

Figure 1 is a schematic representation of one embodiment of the method of the present invention, in which a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site is employed.

Figure 2 is a schematic representation of another embodiment of the method of the present invention, in which a modified oligonucleotide having a region comprising a compound which specifically cleaves single-stranded DNA at a selected site is employed.

Figure 3 is a schematic representation of an embodiment of the method of the present invention, in which a DNA oligonucleotide and a restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site are employed.

Figure 4 is a schematic representation of an embodiment of the method of the present invention, in which a restriction oligonucleotide, a restriction complement oligonucleotide and a restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site at a selected site are employed.

Figure 5 is a schematic representation of an embodiment of the method of the present invention, in which a promoter primer complement oligonucleotide is employed.

Figure 6 is a schematic representation of an embodiment of the method of the present invention, in which a restriction oligonucleotide and a restriction

enzyme which recognizes single-stranded DNA and specifically cleaves outside its recongition site at a selected site are employed.

Figure 7 is a schematic representation of an embodiment of the method of the present invention, in which RNA of interest is amplified.

Figure 8 is a schematic representation of an embodiment of the method of the present invention, in which a DNA-dependent RNA polymerase which recongizes single-stranded DNA and nonspecifically snythesizes RNA is employed.

Figure 9 is a schematic representaion of the method of the present invention whereby a region of the ampicillin resistance gene is amplified.

Detailed Description of the Invention

The present invention pertains to a method for the amplification of RNA or DNA. Through use of the method, the quantity of selected DNA or RNA (referred to, respectively, as DNA of interest and RNA of interest) is increased as desired by combining the necessary reagents and maintaining the resulting combination under appropriate conditions. The present method is particularly useful because it is not a multi-step procedure (i.e., does not require sequential combination of reagents in a predetermined order critical to the success of the method).

The DNA or RNA of interest to be amplified can be only a portion of a longer sequence, referred to, respectively, as a target DNA or target RNA sequence, or can constitute the entire target DNA or target RNA sequence (in which case, the DNA of interest or RNA of interest and the target DNA or target RNA sequence,

respectively, are the same). The target DNA or RNA sequence can be obtained from various sources, such as from cloned DNA, from plasmids or obtained from various naturally-occuring sources, including bacteria, yeast, viruses, and higher organisms such as plants or animals, in purified or non-purified form.

In the amplification method of the present invention, DNA or RNA to be amplified, which is DNA of interest having a defined 3' end or RNA of interest, is combined with the following reagents: two single-stranded DNA primers; a RNA-dependent DNA polymerase; a DNA-dependent DNA polymerase; a DNA-dependent RNA polymerase; an enzyme or an agent with an RNase H activity; (i.e., an enzyme or an agent which has the ability to degrade the RNA strand of a RNA/DNA heteroduplex); and appropriate nucleoside triphosphates.

The two DNA primers used are referred to as a promoter primer and a reverse primer. The promoter primer is an oligonucleotide which includes at least two regions whose presence is necessary for the promoter primer to be useful in the present method: a 5' region which includes a recognition sequence for a DNA-dependent RNA polymerase and a 3' region which includes a sequence which is complementary to a region of the DNA of interest or the RNA of interest.

DNA sequences may be inserted into the sequence of the promoter primer between the 5' end containing the DNA-dependent RNA polymerase promoter sequence and the 3' end which is complementary to the DNA or RNA of interest. This makes it possible to include additional DNA sequences, adjacent to the DNA or RNA of interest which would not normally be found there.

When the DNA or RNA of interest is amplified, a DNA fragment will be produced which has both the target DNA sequence, the DNA-dependent RNA polymerase promoter sequence, and the additional sequence incorporated into one continuous sequence.

The reverse primer is an oligonucleotide whose sequence is homologous to a region of the DNA of interest or the RNA of interest which is 5' to and nonoverlapping with the region of the DNA of interest or RNA of interest to which the promoter primer is complementary. The reverse primer may also have additional selected sequence at its 5', end which will be incorporated into the resultant DNA fragment during the amplification process. As a result, the reverse primer is capable of hybridizing to a RNA strand or a DNA strand which is complementary to the RNA of interest or the DNA of interest. DNA of interest must be processed in order for it to become a substrate for amplification. One method of processing is to make a complementary RNA copy of the DNA of interest, which is able to react directly in the amplification reaction. Methods for accomplishing this are described below. Another method for processing DNA of interest is to create a 3' defined end in that DNA of interest. DNA of interest which has a defined 3' end is defined as DNA of interest which terminates at its 3' end in a sequence which is sufficiently complementary to the 3' end of the promoter primer that a stable hybrid will be formed when the DNA of interest and a selected promoter primer are combined and maintained under appropriate conditions (e.g., the reaction temperature). The 3' end of the DNA of interest must not extend beyond the region to which

the promoter primer sequence is complementary. There are several methods of processing target DNA so as to create DNA of interest having the defined 3' end which is necessary for the present method; these are described below.

As mentioned above, the subject method requires reagents which act as a RNA-dependent DNA polymerase; a DNA-dependent DNA polymerase; a DNA-dependent RNA polymerase; and an enzyme or an agent with RNase H activity. Each of these four functions can be carried out by a separate reagent; in this case, four different reagents are used to provide the desired activities. However, two or more of these functions can be carried out by one reagent; in this case, three or fewer reagents are needed. For example, the enzyme reverse transcriptase can function as a RNA-dependent DNA polymerase and as a DNA-dependent DNA polymerase and can also exhibit an RNase H-like activity.

The appropriate nucleoside triphosphates which include the necessary DNA and RNA precursors for amplification of target DNA are also included in the reaction mixture in adequate amounts. These include the deoxyribonucleoside 5'-triphosphates dATP, dCTP, dGTP and TTP and the ribonucleoside triphosphates ATP, CTP, GTP, and UTP. In addition, a molar excess of the oligonucleotide primers (as compared to target RNA or DNA present) is added to the buffer containing the target DNA or RNA.

Once the necessary reagents and the target DNA or target RNA are combined, they are maintained under conditions (e.g., time, temperature) appropriate for the reagents to function and amplification to occur. In the case in which DNA of interest is being

amplified, the method proceeds as follows: the 3' end of the promoter primer hydridizes to the defined 3' end of the DNA of interest. Through the action of a DNA-dependent DNA polymerase, the promoter primer is extended in the 3' direction, resulting in production of a sequence complementary to the DNA of interest, thus making the DNA of interest double-stranded. Through the action of the DNA-dependent DNA polymerase, the DNA of interest is also extended from its 3' end, resulting in a sequence complementary to the RNA promoter sequence and, thus, making the RNA promoter region of the promoter primer double-stranded. The double-stranded promoter-DNA of interest product is used as a template by the DNA-dependent RNA polymerase to produce RNA molecules which have a defined 5' end and are complementary to the DNA of interest. The reverse primer hybridizes to the RNA strand at its site of complementarity (i.e., to a nonoverlapping region 3' of the region on the RNA strand to which the promoter primer is homologous). The reverse primer is extended by an RNA-dependent DNA polymerase. An agent with RNase H-like activity (i.e., a substance which hydrolyzes RNA from an RNA/DNA heteroduplex) degrades the RNA strand from the newly formed RNA/DNA heteroduplex, producing a single-stranded DNA fragment with both a defined 3' end and a defined 5' end. The defined 3' end of the DNA fragment is able to hybridize to the 3' end of the promoter primer. The 3' end of the promoter primer is extended by a DNA-dependent DNA polymerase, which produces a complementary copy of the DNA fragment making that region double-stranded. The 3' end of the DNA fragment is extended through the activity of the

DNA-dependent DNA polymerase and, as a result, the RNA promoter region is made double-stranded. The resulting double-stranded DNA fragment is recognized by a DNA-dependent RNA polymerase which synthesizes RNA molecules having a defined 5' and 3' end which are complementary to the target "DNA of interest". RNA molecules having a defined 5' and a defined 3' end can be amplified using the promoter primer, reverse primer and reagents, as described above.

Amplification of RNA of interest by the present method proceeds as follows: The 3' end of the selected promoter primer hybridizes to its complementary region of the RNA of interest. By the action of a RNA-dependent DNA polymerase, the promoter primer is extended from its 3' end. This results in production of DNA complementary in sequence to that of the RNA of interest. An agent with an RNase H activity degrades the RNA strand of the RNA/DNA duplex leaving a single-stranded DNA with the 5' end defined by the promoter primer. The reverse primer hybridizes to a region 3' to and nonoverlapping with the promoter primer sequence contained at the 5' defined end of this single-stranded DNA and is extended in the 3' direction through the action of a DNA-dependent DNA polymerase, to produce a double-stranded DNA molecule which includes the RNA promoter sequence and the double-stranded DNA copy of the target RNA. The double-stranded DNA molecule which includes the RNA promoter sequence is used by a DNA-dependent RNA polymerase to produce RNA molecules with a defined 5' end which is complementary to the original target RNA strand. The reverse primer hybridizes to the RNA strand at its site of complementarity, 3' to and

nonoverlapping with the region of promoter primer homology. The reverse primer is extended by a RNA dependent-DNA polymerase. An agent with RNase H-like activity (e.g., which hydrolyzes RNA from an RNA/DNA heteroduplex) degrades the RNA strand from the newly formed RNA/DNA heteroduplex. This step leaves a single-stranded DNA sequence with a defined 3' end and a defined 5' end. The defined 3' end of the DNA sequence is able to hybridize to the 3' end of the promoter primer and the 3' end of the promoter primer is extended by a DNA-dependent DNA polymerase, producing a complementary copy of the DNA sequence. In addition, the 3' end of the DNA sequence is extended using the DNA-dependent DNA polymerase. This produces a double-stranded DNA molecule containing the RNA promoter sequence and DNA sequence of interest. This double-stranded DNA fragment is recognized by a DNA-dependent RNA polymerase, which produces a RNA sequence with a defined 5' and 3' end. This RNA sequence can be amplified using the promoter primer and reverse primer as described above.

Several embodiments of the amplification method of the present invention are described in detail in the following sections. In each embodiment, a target DNA sequence in which DNA of interest is available for hybridization is combined with the reagents necessary for amplification to be carried out. The reagents used in each embodiment are described below.

In one embodiment, a restriction enzyme which recognizes and cleaves single-stranded DNA at a selected site is employed (see Figure 1). The restriction enzyme is combined, under appropriate conditions, with a target DNA sequence; a promoter

primer; a reverse primer; at least one RNA-dependent DNA polymerase; at least one DNA-dependent DNA polymerase; at least one DNA-dependent RNA polymerase; an agent with RNase H activity; and appropriate nucleoside triphosphates. When combined under appropriate conditions (e.g., appropriate temperature and pH, etc.), amplification of the DNA of interest occurs spontaneously. The target DNA sequence is cleaved at a selected site by the restriction enzyme, which recognizes and specifically cleaves single-stranded DNA, to produce DNA of interest having a defined 3' end. Subsequently, a 3' region of the promoter primer hybridizes to the complementary 3' end of the DNA of interest. The 3' end of the promoter primer is extended by the DNA-dependent DNA polymerase, using the DNA of interest as a template, and the 3' end of the DNA of interest is extended by the DNA-dependent DNA polymerase, using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter.

Subsequently, this double-stranded DNA sequence is transcribed by the DNA-dependent RNA polymerase to produce a RNA transcript having a defined 5' end. The reverse primer hybridizes to a complementary 3' region of the RNA transcript and the 3' end of the reverse primer is extended by the RNA-dependent DNA polymerase, using the RNA transcipt as a template. The product of this extension process is a heteroduplex molecule comprising the RNA transcript having a defined 5' end and a reverse primer extension product having a sequence which corresponds to the DNA of interest.

The RNA transcript having a defined 5' end and hybridized to the reverse primer extension product is hydrolyzed by the agent with RNase H activity. Subsequently, a 3' region of the promoter primer hybridizes to a complementary 3' region of the reverse primer extension product. The 3' end of the promoter primer is extended by the DNA-dependent DNA polymerase, using the reverse primer extension product as a template and the 3' end of the reverse primer extension product is extended by the DNA-dependent DNA polymerase, using the promoter primer as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter. The double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter is transcribed by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest. In the method of the present invention, this RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest is used as a template in additional amplification cycles, thereby resulting in amplification of the DNA of interest.

In addition to the amplification method described above employing a restriction enzyme, there are other methods for processing the target DNA sequence to produce DNA of interest having a defined 3' end available for hybridization. For example, in another embodiment, represented schematically in Figure 2, a modified oligonucleotide is used to produce DNA of interest having a defined 3' end available for hybridization. The modified oligonucleotide is

complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest. The 3' end of the oligonucleotide is comprised of a compound which is capable, under appropriate conditions, of cleaving single-stranded DNA. In this embodiment, the modified oligonucleotide hybridizes to the complementary region of the target DNA sequence and the compound is activated, allowing it to specifically cleave the single-stranded DNA target DNA sequence at the selected site, to produce DNA of interest having a defined 3' end available for hybridization. This DNA of interest having a defined 3' end is amplified using the primers and enzymes described above, in an isothermal, cyclic amplification reaction.

Alternatively, an oligonucleotide and a restriction enzyme are used to produce DNA of interest having a defined 3' end available for hybridization. The oligonucleotide is complementary to a region of DNA of interest comprising a sequence which corresponds to the recognition site of a restriction enzyme which is capable of recognizing and specifically cleaving double-stranded DNA at a selected site adjacent to the defined 3' end of the DNA of interest. In this embodiment, represented schematically in Figure 3, the oligonucleotide hybridizes to the complementary region of the target DNA sequence and the enzyme which specifically cleaves double-stranded DNA cleaves the target DNA sequence at a selected site, to produce DNA of interest having a defined 3' end available for hybridization. This DNA of interest having a defined 3' end is amplified using a promoter primer, a reverse primer and the enzymes described

above, according to the amplification method of the present invention.

Another embodiment of the method of the present invention, represented in Figure 4, utilizes a restriction oligonucleotide, a restriction complement oligonucleotide and a restriction enzyme to produce DNA of interest having a defined 3' end available for hybridization. The restriction oligonucleotide has a 3' region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of a restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site. In this embodiment, the restriction oligonucleotide is combined with a restriction complement oligonucleotide having a region complementary to a 5' region of the restriction oligonucleotide. The restriction complement oligonucleotide hybridizes to the 5' region of the restriction primer to form a restriction complex having a double-stranded recognition site for the enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site. In addition, the restriction complex has a region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest. The restriction complex hybridizes to the target DNA sequence and the restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site cleaves the target DNA sequence at a selected site, to produce DNA of interest having a defined 3' end available for hybridization. As

described above, this DNA of interest is combined with a promoter primer, a reverse primer and the selected enzymes described above, to amplify the DNA of interest in an isothermal, cyclic reaction.

In yet another embodiment, a promoter-complement primer, which has a region complementary to the promoter sequence of the promoter primer described above, is used in the amplification method, to amplify the DNA of interest contained in target DNA. In this embodiment, represented in Figure 5, the promoter-complement primer is combined with the target DNA sequence, the promoter primer, the reverse primer, and the selected enzymes described above. The promoter-complement primer hybridizes to the promoter sequence of the promoter primer, to produce a double-stranded promoter complex. The double-stranded promoter complex hybridizes to the 3' end of the DNA of interest. The 3' end of the promoter primer is extended by the DNA-dependent DNA polymerase using the DNA of interest as a template, to produce a double-stranded DNA sequence having a double-stranded promoter. This double-stranded DNA sequence is an intermediate in the amplification process described above, which proceeds to result in amplification of the DNA of interest.

Alternatively, a restriction primer is used to produce DNA of interest having a defined 3' end available for hybridization. The restriction primer has a 3' region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of a restriction enzyme which recognizes single-stranded

DNA and specifically cleaves outside its recognition site at a selected site. The restriction primer is combined with the target DNA sequence, a promoter primer, a reverse primer, and the enzymes described above to amplify the DNA of interest. In this embodiment, represented in Figure 6, the 3' region of the restriction primer hybridizes to the complementary region of the target DNA sequence. The target DNA sequence is cleaved at a selected site by the restriction enzyme, which recognizes single- stranded DNA and specifically cleaves outside its recognition site, to produce DNA of interest having a defined 3' end available for hybridization. This DNA of interest having a defined 3' end is amplified in the isothermal, cyclic reaction described above.

In a further embodiment of the method of the present invention, which is represented in Figure 7, the target DNA sequence is combined with a DNA-dependent RNA polymerase which recognizes single-stranded DNA and nonspecifically synthesizes RNA using the DNA of interest as a template. In this method, a RNA/DNA heteroduplex molecule is produced by the DNA-dependent RNA polymerase. Subsequently, a RNA sequence is produced by the DNA-dependent RNA polymerase using the RNA/DNA heteroduplex as a template. In addition, the RNA sequence synthesized using the DNA of interest as a template is combined with two primers; a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to a 3' region of the RNA sequence synthesized using the DNA of interest as a template; and a reverse primer having a region complementary to

the 3' end of the DNA of interest. Additionally, the above-described enzymes and appropriate nucleoside triphosphates are included in the reaction mixture, to result in amplification of the DNA of interest according to the method of the present invention.

The term "DNA of interest" as used herein refers to DNA contained within a target DNA sequence, which can be either a single-stranded or double-stranded nucleic acid, to be amplified in the method of the present invention. The DNA of interest has both a "defined 3' end" and a "defined 5' end". In addition, the nucleic acid sequence of the 3' end and of the 5' end of the DNA of interest are known in sufficient detail that oligonucleotide primers can be provided which are sufficiently complementary to their respective targets such that hybridization occurs. The defined 3' end of the DNA of interest is that region of the DNA of interest to which a 3' region of a promoter primer is complementary and hybridizes thereto. In some cases, the defined 3' end is defined by cleavage of the target DNA sequence, to produce DNA of interest having a defined 3' end available for hybridization of the promoter primer and subsequent extension of both the promoter primer and the 3' end of the DNA of interest, to produce a double-stranded DNA sequence having a double-stranded promoter.

The DNA of interest is contained within a target DNA sequence which can be a single-stranded or a double-stranded nucleic acid sequence. The DNA of interest can be only a portion of the target DNA sequence. Alternatively, the DNA of interest can be the entire target DNA sequence, such that the terms

"DNA of interest" and "target DNA sequence" refer to the same discrete molecule.

The target DNA sequence is treated, if necessary, to render the DNA of interest available for hybridization. Thus, if the target DNA sequence is initially double-stranded, the strands are separated (e.g., by heat denaturation or enzyme separation) to produce two separate strands. Alternatively, only a portion of the double-stranded target DNA sequence is separated to render the DNA of interest available for hybridization. This can be accomplished by a variety of known enzymes.

The term "oligonucleotide" as used in the method herein in referring to primers, probes, or fragments thereof, is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. The length of the oligonucleotide depends on many factors, which in turn depends on the ultimate function or use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, which occurs naturally or is produced synthetically, and capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced. For example, in the presence of the appropriate nucleoside triphosphates and an inducing agent, such as a DNA-dependent DNA polymerase or a RNA-dependent DNA polymerase, and at a suitable temperature and pH, the primer acts as a point of initiation of synthesis. In addition, the primer can be of various lengths, but must be sufficiently long to prime the synthesis of extension products in the presence of the inducing

agent. Primers useful in the method of the present invention are described in detail in U.S. Patent No. 4,683,202, the contents of which are incorporated herein by reference.

Several primers are used in the various embodiments of the amplification method described herein for processing the target DNA sequence to produce DNA of interest having a defined 3' end and subsequently amplifying the DNA of interest having a defined 3' end. At least two primers are necessary for the amplification process: a "promoter primer" and a "reverse primer", each of which is described above.

The primers used are "sufficiently" complementary to their respective target nucleic acid sequence to be amplified. For example, the promoter primer is sufficiently complementary to the defined 3' end of the DNA of interest contained in a target DNA sequence. That is, the primers must be sufficiently complementary in nucleic acid sequence to the target nucleic acid sequence for hybridization to occur. Thus, the primer sequence need not be the exact complement of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. For example, the promoter primer has a 5' region comprising a DNA-dependent RNA polymerase promoter sequence. In addition, non-complementary sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the nucleic acid sequence of the DNA of interest that hybridization occurs.

The oligonucleotide primers can be produced using any one of various methods, such as, for example, the phosphotriester and phosphodiester methods or automated embodiments thereof. Methods for synthesizing oligonucleotides are described in detail in U.S. Patent No. 4,683,202, the contents of which have been previously incorporated herein. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The term "corresponds to" as used herein refers to nucleic acid sequences which are homologous or substantially homologous. For example, the reverse primer is selected having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template. This RNA transcript is complementary to the DNA of interest. Thus, the reverse primer has a region which "corresponds to", or is substantially homologous to a 5' region of the DNA of interest. The degree of homology depends on many factors, including the degree of complementarity of the reverse primer for the RNA transcript.

As used herein, the term "restriction enzyme" refers to an enzyme capable of specifically cleaving either single-stranded or double-stranded DNA at a selected site (i.e., at or near a specific nucleotide sequence). In one embodiment, a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site is used in the amplification method of the present invention. For example, enzymes which can be used to cleave single-stranded DNA are selected from, but not limited to:

Hha 1, BstN 1, Dde 1, Hae 3, Hga 1, Hinf 1, Hinp 1, Mnl 1, Rsa 1, Taq 1. In addition, an enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site can be used to produce DNA of interest having a defined 3' end. For example, enzymes which can be used to cleave double-stranded DNA are selected from, but not limited to: EcoRl, BamHl, Stul, HindIII, ScaI, HaeIII. In another embodiment, an enzyme which recognizes either single-stranded DNA or double-stranded DNA and specifically cleaves outside its recognition site is used. For example, Fok 1 is an enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site at a selected site. Szybalski, W., Gene, 40:169-173 (1985); Podhajska, A., and Szybalski, W., Gene, 40:175-182 (1985). In addition, Mnl 1 is an enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site.

Alternatively, a non-enzymatic agent or compound which cleaves single-stranded DNA can be attached to a DNA probe to specifically cleave the target DNA sequence at a selected site, to produce DNA of interest having a defined 3' end available for hybridization. Any agent or compound which will function to cleave single-stranded DNA at a selected site can be used in the method of the present invention. For example, ethylenediaminetetracetic acid or diethylenediaminepentacetic acid can be covalently attached to a DNA probe and, in the presence of Fe^{+2} and dithiothreitol, can specifically cleave single-stranded DNA at a selected site. Chu,

B.C.F., and Orgel, L.E., Proc. Natl. Acad. Sci., 82:963-967 (1985); Kazakov, S.A., et al., Nature, 335:186-188 (1988).

An inducing agent, or agent which catalyzes the primer extension reaction can be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. These are referred to herein as DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. Suitable enzymes for this purpose include, but are not limited to, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. In addition, reverse transcriptase can be used interchangeably as a RNA-dependent DNA polymerase and a DNA-dependent DNA polymerase. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the appropriate template (e.g., the DNA of interest) and at the 3' end of the appropriate template and proceed in the 5' direction along the primer, until synthesis terminates in both directions. Inducing agents which initiate synthesis at the 5' end and proceed in the 3' direction can also be used, as described above for those which initiate synthesis at the 3' end.

In addition, a DNA-dependent RNA polymerase is necessary for the isothermal amplification method described herein. The DNA-dependent RNA polymerase recognizes a double-stranded RNA polymerase promoter

sequence and synthesizes a RNA transcript using the appropriate DNA sequence as a template. Some examples of useful RNA polymerases are: T3, T7, SP6. In one embodiment, a T7 RNA polymerase is used which recognizes single-stranded DNA and nonspecifically synthesizes a RNA transcript. The T7 RNA polymerase synthesizes a RNA sequence using a single-stranded DNA sequence as a template, to produce a heteroduplex molecule (Chamberlin, M. and Ring, J., Journal of Biological Chemistry, 248:2235-2244 (1973)). Subsequently, the T7 RNA polymerase uses the heteroduplex molecule as a template, to produce a RNA transcript. This RNA transcript is combined, under appropriate conditions, with the target DNA sequence; a promoter primer; a reverse primer; selected enzymes; and nucleoside triphosphates in the one-pot, isothermal amplification method described herein.

Alternatively, the T7 RNA polymerase can first be combined with a target DNA sequence under conditions appropriate for the synthesis of a RNA transcript, using the target DNA sequence as a template, to produce a heteroduplex molecule. This heteroduplex molecule can be separated by heat denaturation or enzyme separation. Subsequently, the RNA transcript synthesized using the target DNA sequence as a template can be combined with the above-described reagents necessary for the amplification method of the present invention.

An agent with an RNase H-like activity which hydrolyzes RNA present in a RNA/DNA heteroduplex can be any compound which will function to separate a RNA sequence hybridized to a DNA sequence. For example, ribonuclease H (RNase H) specifically degrades RNA

which is hybridized to DNA. When added to the reaction mixture, RNase H avoids the time-consuming task of separate heating and cooling manipulations necessary to denature RNA/DNA heteroduplexes and provides for an isothermal amplification reaction.

The amplification of the DNA of interest occurs under appropriate conditions in a one-pot, isothermal, cyclic, reaction employing a number of selected enzymes and at least two primers. In general, the appropriate conditions for the amplification process to occur include a buffered aqueous solution, preferably at a pH of 7-9, and an appropriate temperature, approximately 37°C.

Specific Embodiments of the Amplification Method of the Present Invention

The following sections describe in detail the isothermal amplification method of the present invention and the various embodiments thereof, for processing the target DNA sequence, to produce DNA of interest having a defined 3' end available for hybridization and subsequently amplifying the DNA of interest.

I. Use of a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site

In the isothermal amplification method of the present invention, a target DNA sequence containing DNA of interest is combined, under appropriate conditions, with at least two primers, several selected enzymes and appropriate nucleoside triphosphates, to amplify the DNA of interest having a defined 3' end.

When the amplification reagents are combined, amplification of the DNA of interest occurs spontaneously. The following is a detailed description of the amplification method of the present invention employing a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site, to produce DNA of interest having a defined 3' end available for hybridization. The method as described in this section can be used in several different embodiments, the variations to which are described in the following sections with reference to the description given herein.

In all of the embodiments described herein, the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization, is combined with the following: a promoter primer; a reverse primer; at least one RNA-dependent DNA polymerase; at least one DNA-dependent DNA polymerase; at least one DNA-dependent RNA polymerase; an agent with RNase H activity (i.e., hydrolyzes RNA present in a RNA/DNA heteroduplex); and appropriate nucleoside triphosphates. The promoter primer is selected having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest. When the 3' region of the promoter primer hybridizes to the 3' end of the DNA of interest, the 5' region comprising a promoter sequence remains single-stranded.

As diagrammed in Figure 1, the present embodiment employs a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site. In the methods employing a restriction enzyme

which specifically cleaves the target DNA sequence at a selected site (i.e., at or near the defined 3' end of the DNA of interest), the restriction enzyme first cleaves the target DNA sequence at a selected site, to produce DNA of interest having a defined 3' end available for hybridization.

After cleavage of the target DNA sequence, the 3' region of the promoter primer hybridizes to the complementary defined 3' end of the DNA of interest. Subsequently, the 3' end of the promoter primer is extended by a DNA-dependent DNA polymerase using the DNA of interest as a template. In addition, the 3' end of the DNA of interest is extended by the DNA-dependent DNA polymerase using the promoter primer as a template. The result of this extension process is a double-stranded DNA sequence having a double-stranded promoter. The double-stranded DNA comprises the DNA of interest extension product, the promoter primer extension product and a double-stranded DNA-dependent RNA polymerase promoter. A DNA-dependent RNA polymerase recognizes the double-stranded promoter region of the double-stranded DNA and transcribes the DNA, to produce a RNA transcript having a defined 5' end. The RNA transcript has a sequence which is complementary to the DNA of interest and has a defined 5' end complementary to the defined 3' end of the DNA of interest (i.e., the 5' end of the RNA transcript is defined by the 3' end of the DNA of interest produced by enzymatic cleavage of the target DNA sequence).

In each of the following embodiments, a reverse primer is necessary for amplification of the DNA of interest. The reverse primer has a region

complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template. This RNA transcript, to which the reverse primer is complementary, is the same RNA transcript having a defined 5' end described above. The reverse primer hybridizes to the complementary 3' region of the RNA transcript having a defined 5' end. The 3' end of the reverse primer is extended by a RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript and a reverse primer extension product having a sequence which is homologous to the DNA of interest. Thus, the reverse primer extension product has a nucleic acid sequence substantially homologous to the DNA of interest. The degree of homology depends on several factors, including the degree of complementarity of the reverse primer for the RNA transcript.

Subsequently, the RNA transcript hybridized to the reverse primer extension product is hydrolyzed by an agent with RNase H activity, which recognizes a RNA sequence hybridized to a DNA sequence and degrades the RNA sequence. After hydrolysis of the RNA transcript, the reverse-primer extension product is available for hybridization with complementary nucleic acids. The reverse primer extension product has a sequence which corresponds to the DNA of interest. Thus, the promoter primer which has a 3' region complementary to the 3' end of the DNA of interest is also complementary to the 3' end of the reverse-primer extension product. The promoter primer hybridizes to the complementary 3' region of the reverse-primer

extension product. Subsequently, the 3' end of the promoter primer is extended by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and the 3' end of the reverse primer extension product is extended by the DNA-dependent DNA polymerase using the promoter- containing primer as a template.

The result of this extension process is a double-stranded DNA sequence having a defined 3' end, a defined 5' end and a double-stranded promoter. This DNA sequence having a double stranded promoter comprises a sequence which corresponds to the DNA of interest (i.e., the reverse primer extension product which is substantially homologous to the DNA of interest) and a sequence which is complementary to the DNA of interest (i.e., the promoter primer extension product). Thus, the 3' end and the 5' end of the double-stranded DNA are defined. What this means is that the 3' end and the 5' end are available for hybridization (i.e., the 3' end and 5' end at a particular nucleic acid). The nucleic acid sequence at the 3' end and the 5' end of the reverse primer extension product corresponds to the sequence at the 3' end and the 5' end of the DNA of interest and the sequence at the 3' end and the 5' end of the promoter primer extension product is complementary to the 3' end and the 5' end of the DNA of interest. In addition, the double-stranded DNA sequence has a double-stranded DNA-dependent RNA polymerase promoter.

The double-stranded DNA sequence having a defined 3' end, a defined 5' end and a double-stranded promoter is transcribed by a DNA-dependent RNA polymerase, which recognizes the double-stranded

promoter thereof, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest. The RNA transcript has a defined 3' end and a defined 5' end complementary to the nucleic acid sequences at the 3' end and the 5' end of the DNA of interest. This RNA transcript is used as a template in additional amplification cycles, thereby resulting in amplification of the DNA of interest.

II. Use of a modified oligonucleotide and a compound which cleaves single-stranded DNA

In another embodiment of the method of the present invention, represented in Figure 2, a modified oligonucleotide is employed. The modified oligonucleotide is selected to be complementary to a region within a target DNA sequence adjacent to the defined 3' end of the DNA of interest with a 3' end comprising a compound which specifically cleaves single-stranded DNA. The modified oligonucleotide hybridizes to the target DNA sequence at a location 3' of the defined 3' end of the DNA of interest. When the modified oligonucleotide hybridizes to the target DNA sequence, the 3' end of the modified oligonucleotide, which comprises the compound which specifically cleaves single-stranded DNA is brought in proximity with the 3' defined end of the target DNA to be cleaved.

In this embodiment, the modified oligonucleotide is combined, under appropriate conditions, with the target DNA sequence; a promoter primer; a reverse primer; the above-described enzymes; and appropriate nucleoside triphosphates. The modified

oligonucleotides hybridizes to the complementary region of the target DNA sequence (i.e., a region adjacent to the defined 3' end of the DNA of interest). Subsequently, the target DNA sequence is cleaved at a selected site by the compound which specifically cleaves single-stranded DNA, to produce DNA of interest having a defined 3' end. The DNA of interest having a defined 3' end is amplified as described above in Section I.

III. Use of a restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site.

In another embodiment, represented in Figure 3, a DNA oligonucleotide is used in combination with a restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site. The DNA oligonucleotide is selected having a 5' region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest and a 3' region comprising a sequence which corresponds to the recognition site of the restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site. The 5' region of the DNA oligonucleotide can hybridize to the target DNA sequence at a location 3' of the defined 3' end of the DNA of interest. When hybridized, the DNA oligonucleotide forms a double-stranded complex which the appropriate restriction enzyme recognizes; the target DNA sequence is cleaved by the restriction enzyme to produce DNA of interest having a defined 3' end available for hybridization.

In this embodiment, the DNA oligonucleotide is combined, under appropriate conditions, with the target DNA sequence; the appropriate restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site; a promoter primer; a reverse primer; the enzymes described above; and appropriate nucleoside triphosphates. The DNA nucleotide hybridizes to the complementary region of the target DNA sequence. Subsequently, the target DNA sequence is cleaved at a selected site by the enzyme which specifically cleaves double-stranded DNA, to produce DNA of interest having a defined 3' end. The DNA of interest having a defined 3' end cleaved by the restriction enzyme is amplified using the method described above in Section I.

IV. Use of a restriction complex and a restriction enzyme which recognizes double-stranded DNA and cleaves outside its recognition site

In yet another embodiment, represented in Figure 4, two additional oligonucleotides are used to produce DNA of interest having a defined 3' end available for hybridization. A restriction oligonucleotide is selected having a 3' region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of a restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site. The 3' region of the restriction oligonucleotide hybridizes to the target DNA sequence at a location 3' of the defined 3' end of the DNA of interest. A second oligonucleotide,

referred to as a restriction complement oligonucleotide, which has a region complementary to the 5' region of the restriction oligonucleotide, hybridizes to the 5' region of the restriction oligonucleotide. When hybridized, the restriction oligonucleotide and the restriction complement primer form a double-stranded complex comprising the recognition site of the restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site.

In this embodiment, the restriction oligonucleotide and the restriction complement oligonucleotide are combined, under appropriate conditions, with the target DNA sequence; the appropriate restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site; a promoter primer; a reverse primer; the above-described enzymes; and appropriate nucleoside triphosphates. The restriction complement oligonucleotide hybridizes to the 5' region of the restriction oligonucleotide to form a restriction complex having a double-stranded recognition site for the enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site. The restriction complex also has a region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest. The restriction complex hybridizes to the target DNA sequence. Subsequently, the target DNA sequence is cleaved at a selected site by the restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site, to produce DNA of interest having a

defined 3' end. This DNA of interest is amplified according to the method described in Section I above.

V. Use of a promoter primer complement oligonucleotide

In an alternative embodiment, represented in Figure 5, a promoter primer complement oligonucleotide is selected having a region complementary to the promoter sequence of the promoter primer. This oligonucleotide is combined, under appropriate conditions, with the target DNA sequence; a promoter primer; a reverse primer; the above-described enzymes; and appropriate nucleoside triphosphates. In this method, the promoter primer complement oligonucleotide hybridizes to the promoter sequence of the promoter primer, to produce a double-stranded promoter complex. Subsequently, the double-stranded promoter complex hybridizes to the defined 3' end of the DNA of interest. The 3' end of the promoter primer is extended by a DNA-dependent DNA polymerase using the DNA of interest as a template, to produce a double-stranded DNA sequence having a double-stranded promoter. A DNA-dependent RNA polymerase recognizes the double-stranded promoter region and transcribes the double-stranded DNA sequence, to produce a RNA transcript having a defined 5' end.

Subsequently, a reverse primer having a region complementary to a 3' region of the RNA transcript having a defined 5' end hybridizes to the RNA transcript. The 3' end of the reverse primer is extended by a RNA-dependent DNA polymerase, to produce a heteroduplex molecule. This heteroduplex molecule comprises the RNA transcript and a reverse primer

extension product having a sequence which corresponds to the DNA of interest. The RNA transcript hybridized to the reverse primer extension product is hydrolyzed by an agent with RNase H activity. The hydrolysis of the RNA transcript leaves the reverse primer extension product available for hybridization. The reverse primer extension product has a sequence which corresponds to the DNA of interest. Thus, the 3' region of the promoter primer hybridizes to a complementary 3' region of the reverse primer extension product. The 3' end of the promoter primer is extended by a DNA-dependent DNA polymerase using the reverse primer extension product as a template and the 3' end of the reverse primer extension product is extended by the DNA-dependent DNA polymerase using the promoter primer as a template. The result of this extension process is a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter. This double-stranded DNA sequence is transcribed by a DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest. This RNA transcript is used as a template in additional amplification cycles, thereby resulting in amplification of the DNA of interest.

VI. Use of a restriction oligonucleotide and a restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site

In an embodiment of the amplification method of the present invention, represented in Figure 6, a

restriction oligonucleotide is employed in combination with a restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site. The restriction oligonucleotide is selected having a 3' region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of the restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site. The 3' region of the restriction oligonucleotide can hybridize to the target DNA sequence at a location 3' of the defined 3' end of the DNA of interest. When hybridized, the restriction oligonucleotide forms a complex which the restriction enzyme recognizes and cleaves outside its recognition site to cleave the target DNA sequence at a selected site.

In this embodiment, the target DNA sequence is combined, under appropriate conditions, with the restriction oligonucleotide; the appropriate restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site; a promoter primer; a reverse primer; the above-described enzymes; and appropriate nucleoside triphosphates. The 3' region of the restriction oligonucleotide hybridizes to the complementary region of the target DNA sequence. Subsequently, the target DNA sequence is cleaved at a selected site by the restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site, to produce DNA of interest having a defined 3' end available for

hybridization. The DNA of interest having a defined 3' end is subsequently amplified according to the method described above in Section I.

VII. Use of a DNA-dependent RNA polymerase

In an alternate embodiment, the target DNA sequence is combined, under appropriate conditions, with a DNA-dependent RNA polymerase which recognizes single-stranded DNA and nonspecifically synthesizes RNA, using the DNA of interest as a template. The result is that a RNA/DNA heteroduplex molecule is produced. Subsequently, single-stranded RNA is produced by the DNA-dependent RNA polymerase, using the RNA/DNA heteroduplex as a template.

In this embodiment, represented in Figure 8, the RNA transcript synthesized using the DNA of interest as a template is combined with two selected primers, the enzymes described above, and appropriate nucleoside triphosphates. One of the primers is a promoter primer which has a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to a 3' region of the RNA sequence synthesized using the DNA of interest as a template. The second primer is a reverse primer which has a region complementary to the 3' end of the DNA of interest. In this method, the promoter primer hybridizes to the complementary 3' region of the RNA sequence synthesized by the DNA-dependent RNA polymerase using the DNA of interest as a template. The 3' end of the promoter primer is extended by a RNA-dependent DNA polymerase, using the RNA sequence as a template, to produce a heteroduplex molecule comprising the RNA sequence and a promoter

primer extension product having a sequence which corresponds to the DNA of interest. Subsequently, the RNA sequence is hydrolyzed by an agent which catalyzes the hydrolysis of RNA present in a RNA/DNA heteroduplex.

After hydrolysis of the RNA transcript, the promoter primer extension product is available for hybridization with complementary nucleic acids. The promoter primer extension product has a sequence which corresponds to the DNA of interest. Thus, the reverse primer which has a 3' region complementary to the 3' end of the DNA of interest is also complementary to the 3' end of the promoter primer extension product. The 3' region of the reverse primer hybridizes to the complementary 3' region of the promoter primer extension product. The 3' end of the reverse primer is extended by a DNA-dependent DNA polymerase, using the promoter primer extension product as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter. This double-stranded DNA sequence is transcribed by a DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence which corresponds to the DNA of interest. This RNA transcript is used as a template in additional amplification cycles, thereby resulting in amplification of the DNA of interest.

Uses of the Isothermal Amplification Method of the Present Invention

The present invention pertains to a one-pot, isothermal method for amplifying DNA of interest which has a defined 3' end and is contained in a target DNA

sequence. The amplification method of the present invention results in an increase in the amount of DNA of interest or RNA of interest. Thus, the present invention can be used for improving the efficiency of cloning DNA of interest contained in a target DNA sequence and for amplifying DNA of interest contained in a target DNA sequence to facilitate detection and/or identification.

For example, the present method herein can be used to enable detection and/or characterization of DNA of interest associated with pathogens, infectious diseases, genetic disorders and cellular disorders. Amplification is useful when the amount of DNA of interest available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Genetic diseases can include specific deletions and/or mutations in genomic DNA from an organism. For example, sickle cell anemia, cystic fibrosis, α-thalassemia, β-thalassemia, and the like. These genetic diseases can be detected by amplifying the appropriate DNA of interest and analyzing it by Southern blots without using radioactive probes. Methods of analyzing isolated DNA of interest and genetic disorders, cellular disorders and infectious diseases of interest are described in detail in U.S. Patent No. 4,683,202, the contents of which have been previously incorporated.

Various infectious diseases can be diagnosed by the presence in clinical samples of DNA of interest characteristic of the pathogen. These include bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis viruses; and

protozoan parasites, such as the Plasmodium responsible for malaria.

The method herein can be utilized to clone DNA of interest for insertion into a suitable expression vector. The vector can then be used to transform an appropriate host organism to produce the gene product of the DNA of interest by standard methods of recombinant DNA technology.

In another embodiment, a small sample of DNA of interest can be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which can be analyzed by restriction and electrophoretic separation or any other appropriate method.

In addition, the process herein can be used for in vitro mutagenesis. The primers used in the amplification process (i.e., the promoter primer and reverse primer) need not be exactly complementary to the DNA of interest which is being amplified. It is only necessary that they be sufficiently complementary to hybridize to their respective targets to be extended by the inducing agent employed. The product of an amplification reaction wherein the primers employed are not exactly complementary to the original template will contain the sequence of the primer rather than the template, thereby introducing an in vitro mutation. In further cycles, this mutation will be amplified with an undiminished efficiency because no further mispaired primings are required. The product having the mutation can be inserted into an

appropriate vector by standard techniques for production of an altered protein.

EXEMPLIFICATION

The following bacterial strains were picked from frozen stocks and innoculated into 3 mls of LB broth with and without 50 μg/ml ampicillin:

1. Strain A - no plasmid
2. Strain B - no plasmid
3. Strain C - pUC 8 plasmid
4. Strain D - pUC 13 plasmid

Both the pUC 8 and the pUC 13 plasmids contain the ampicillin resistance gene to which the primers for the PCR and the amplification method of the present invention (MEA or multiple enzyme amplification) are directed. The innoculated tubes were shaken at 37°C overnight. Growth of the various cultures were scored as follows:

		LB	LB + Ampicillin
1.	Strain A - no plasmid	+	-
2.	Strain B - no plasmid	+	-
3.	Strain C - pUC 8 plasmid	+	+
4.	Strain D - pUC 13 plasmid	+	+

1.5 mls from the LB only tubes containing bacterial strains A and B and 1.5 mls from the LB + ampicillin tubes containing bacterial strains C and D were transferred to 1.5 ml eppendorf tubes and spun for 30 seconds in a microfuge. The supernatant was removed

and the pellets resuspended in 100 µls of 10 mM Tris/10 mM EDTA pH 8.0 (TE) and left at room temperature for 5 minutes. 200 µls of 0.2 N NaOH/1%SDS solution was added, the tubes mixed by inverting, and left for 5 minutes at room temperature. 150 µls of 3 M potassium acetate was added and the tubes were vortexed and set on ice for 5 minutes. 300 µls of buffered phenol solution was added, the tubes vortexed and then spun in the microfuge for 2 minutes. The aqueous phase was removed to a new 1.5 ml eppendorf tube to which 1 ml of 95% ethanol was added. The tubes were spun in the microfuge for 2 minutes. The pellets were washed once with 70% ethanol and allowed to dry under vacuum. The pellets were resuspended in 25 µls of TE. Each miniprep was then treated with Sca 1 restriction enzyme which would cut the plasmid, if present, in the ampicillin resistance gene giving the desired 3' defined end for the DNA of interest necessary for the amplification method of the present invention. The final volume for each reaction mixture was 50 µls. Each reaction was stopped by adding 5 µls of 0.5 M EDTA to each tube. 5 µls from each reaction was run on a 1% agarose gel along with cesium chloride purified pUC 13 which had been cut with Sca 1 restriction enzyme. The gel was stained with ethidium bromide and scored for the presence of plasmid DNA by eye under ultraviolet light.

Miniprep	Plasmid Present
1. Strain A	-
2. Strain B	-
3. Strain C	+

4. Strain D +++

Neither the strain A nor B minipreps was observed to contain plasmids. Both the strain C and D minipreps contained a plasmid with the strain D miniprep plasmid (pUC 13) being present at a much higher concentration than the strain C miniprep plasmid (pUC 8). Each one of the miniprep tubes was diluted by 1000 fold in distilled water and 5 μls of that dilution was amplified by the PCR or the present amplification method. The sequences of the primers used for both the PCR and MEA reactions were for the promoter primer:

ATTAATACGACTCACTATAGGGAGACCCACTCACCAGTCACAGAA

T7 promoter complementary region

Reverse primer:

CTCCATGGTTATGGCAG

These two primers hybridize to two separate regions in the ampicillin resistance gene and are capable of amplifying that gene in either a PCR reaction or MEA reaction, producing a DNA fragment 104 bp in length. This procedure is diagrammed in Figure 9.

The PCR reaction volume was 50 μls and had a final concentration of 10 mM Tris pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM each of dATP, dCTP, dGTP and TTP; 0.2 μM each primer and 5 units of Taq polymerase (New England Biolabs). The PCR reaction was run for 20

cycles consisting of 30 seconds at 95°C, 30 seconds at 37°C, and 3 minutes at 65°C.

The MEA reaction was run in a volume of 40 µls consisting of 45 mM Tris pH 8.0, 12.5 mM NaCl, 37.5 mM KCl, 1 mM spermidine, 2 mM each of ATP, CTP, GTP, and UTP; 0.5 mM each of dATP, dCTP, dGTP, and TTP; 0.25 µM each primer, 200 units of M-MLV reverse transcriptase (Bethesda Research Labs), and 60 units of T7 RNA polymerase (New England Biolabs). The reverse transcriptase and the T7 RNA polymerase were added after the tubes containing the rest of the reagents were heated to 94°C for 2 minutes and then cooled to 37°C for 30 seconds in order to allow the primers to hybridize to the target DNA. The MEA reaction was allowed to sit at 37°C for 3 hours. As a control, approximately 10^7 molecules of Saul digested cesium chloride pruified pUc13 plasmid was run in the PCR and MEA reactions. This was approximately the same amount of pUC 13 plasmid DNA that was contained in the strain D miniprep as determined by comparing bands on an agarose gel. All reactions were stopped by adding EDTA to a final concentration of 50 mM. 5 µls from each reaction was run on a 10% acrylamide gel. The gel was stained with ethidium bromide and scored for the presence of a band corresponding to 104 base pairs by eye.

	20 cycles PCR	MEA
1. 10^7 molecules purified pUC 13	++	+++
2. Strain A (no plasmid)	-	-
3. Strain B (no plasmid)	-	-
4. Strain C (pUC 8)	+-	+
5. Strain D (pUC 13)	++	+++

The results indicate that with this experiment a 3 hour isothermal MEA reaction was able to amplify target DNA molecules from a biological sample to a greater extent than the PCR procedure was able to do in 20 cycles.

CLAIMS

1. A method for amplifying DNA of interest having a defined 3' end and contained within a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest;

c) a reverse primer having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

d) a restriction enzyme which recognizes and specifically cleaves single-stranded DNA at a selected site;

e) at least one RNA-dependent DNA polymerase;

f) at least one DNA-dependent DNA polymerase;

g) at least one DNA-dependent RNA polymerase;

h) an agent with RNase H activity; and

i) appropriate nucleoside triphosphates, under conditions appropriate for:

1) cleavage of the target DNA sequence at a selected site by the restriction enzyme which recognizes and specifically cleaves single-stranded DNA, to produce DNA of interest having a defined 3' end;

2) hybridization of the 3' region of the promoter primer to the complementary 3' defined end of the DNA of interest;

3) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template and extension at the 3' end of the DNA of interest by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

4) transcription of the double-stranded DNA sequence of (3) by the DNA-dependent RNA polymerase, to produce a RNA transcript complementary to the DNA target sequence and having a defined 5' end;

5) hybridization of the reverse primer to the complementary 3' region of the RNA transcript of (4);

6) extension of the 3' end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (5) and a reverse primer extension product having a sequence which is homologous to the DNA of interest;

7) hydrolysis of the RNA transcript of (6) by the agent with RNase H activity;

8) hybridization of the 3' region of the promoter primer to a complementary 3' region of the reverse primer extension product of (6);

9) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3' end of the reverse primer extension product by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter; and

10) transcription of the double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

2. A method of Claim 1 wherein the agent with RNase H activity is ribonuclease H.

3. A method of Claim 1 wherein the restriction enzyme which recognizes and cleaves single-stranded DNA at a selected site is selected from the group consisting of: Hha 1, BstN 1, Dde 1, Hae 3, Hga 1, Hinf 1, Hinp 1, Mnl 1, Rsa 1, Taq 1.

4. A method for amplifying DNA of interest having a defined 3' end and contained within a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest;

c) a reverse primer having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

d) a modified oligonucleotide which is complementary to a region of the target DNA which is 5' and adjacent to the 3' defined end which is modified at the 5' end with compound capable of cleaving single-stranded DNA when activated;

e) at least one RNA-dependent DNA polymerase;

f) at least one DNA-dependent DNA polymerase;

g) at least one DNA-dependent RNA polymerase;

h) an agent with RNase H activity; and

i) appropriate nucleoside triphosphates,

under conditions appropriate for:

1) hybridization of the modified oligonucleotide to the complementary region of the target DNA sequence;

2) cleavage of the target DNA sequence at a selected site by the compound which specifically cleaves single-stranded DNA, to

produce DNA of interest having a defined 3' end;

3) hybridization of the 3' region of the promoter primer to the complementary 3' defined end of the DNA of interest;

4) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template and extension of the 3' end of the DNA of interest by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

5) transcription of the double-stranded DNA sequence of (4) by the DNA-dependent RNA polymerase, to produce a RNA transcript which is complementary to the target DNA and having a defined 5' end;

6) hybridization of the reverse primer to the complementary 3' region of the RNA transcript of (5);

7) extension of the 3' end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (6) and a reverse primer extension product having a sequence which homologous to the DNA of interest;

8) hydrolysis of the RNA transcript of (7) by the agent with RNase H activity;

9) hybridization of the 3' region of the promoter primer to a complementary 3' region of the reverse primer

extension product of (7);

10) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3' end of the reverse primer extension product by the DNA- dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter; and

11) transcription of the double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

5. A method of Claim 4 wherein the agent with RNase H activity is ribonuclease H.

6. A method of Claim 4 wherein the compound which specifically cleaves single-stranded DNA at a selected site is selected from the group consisting of ethylenediaminetetracetic acid and diethylenetriaminepentacetic acid in the presence of Fe^{2+} and dithiothreitol.

7. A method for amplifying DNA of interest having a defined 3′ end and contained within a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5′ region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3′ of the promoter sequence complementary to the defined 3′ end of the DNA of interest;

c) a reverse primer having a region complementary to a 3′ region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

d) a restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site;

e) a DNA oligonucleotide which is complementary to a region within the target DNA sequence adjacent to and partially overlapping with the defined 3′ end of the DNA of interest which contains a 3′ region comprising a sequence which corresponds to the recognition site of the restriction enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site;

f) at least one RNA-dependent DNA polymerase;

g) at least one DNA-dependent DNA polymerase;

h) at least one DNA-dependent RNA polymerase;

i) an agent with RNase H activity; and

j) appropriate nucleoside triphosphates,

under conditions appropriate for:

1) hybridization of the DNA oligonucleotide to the complementary region of the target DNA sequence;

2) cleavage of the target DNA sequence at a selected site by the enzyme which specifically cleaves double-stranded DNA, to produce DNA of interest having a defined 3' end;

3) hybridization of the 3' region of the promoter primer to the complementary 3' end of the DNA of interest;

4) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template and extension of the 3' end of the DNA of interest by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

5) transcription of the double-stranded DNA sequence of (4) by the DNA-dependent RNA polymerase, to produce a RNA transcript complementary to the target DNA and having a defined 5' end;

6) hybridization of the reverse primer to the complementary 3' region of the RNA transcript of (5);

7) extension of the 3' end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (6) and a reverse

primer extension product having a sequence which is homologous to the DNA of interest;

8) hydrolysis of the RNA transcript of (7) by the agent with RNase H activity;

9) hybridization of the 3' region of the promoter primer to a complementary 3' region of the reverse primer extension product of (7);

10) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3' end of the reverse primer extension product by the DNA- dependent DNA polymerase using the promotercontaining primer as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter; and

11) transcription of the double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

8. A method of Claim 7 wherein the agent with RNase H activity is ribonuclease H.

9. A method of Claim 7 wherein the enzyme which recognizes and specifically cleaves double-stranded DNA at a selected site is selected from the group consisting of: EcoR1, BamH1, Stu1, HindIII, ScaI, HaeIII.

10. A method for amplifying DNA of interest having a defined 3' end and contained within a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest;

c) a reverse primer having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

d) a restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site at a selected site;

e) a restriction oligonucleotide having a 3' region complementary to a region within the target DNA sequence adjacent to and partially overhapping with the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of the restriction enzyme which recognizes

double-stranded DNA and specifically cleaves outside its recognition site;

f) a restriction complement oligonucleotide having a region complementary to the 5' region of the restriction oligonucleotide;

g) at least one RNA-dependent DNA polymerase;

h) at least one DNA-dependent DNA polymerase;

i) at least one DNA-dependent RNA polymerase;

j) an agent with RNase H activity; and

k) appropriate nucleoside triphosphates,

under conditions appropriate for:

1) hybridization of the restriction complement oligonucleotide to the 5' region of the restriction oligonucleotide to form a restriction complex having a double-stranded recognition site for the enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site and a region complementary to a region within the target DNA sequence adjacent to the defined 3' end of the DNA of interest;

2) hybridization of the restriction complex to the target DNA sequence;

3) cleavage of the target DNA sequence at a selected site by the restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site, to produce DNA of interest having a defined 3' end;

4) hybridization of the 3' region of the promoter primer to the complementary 3' end of the DNA of interest;

5) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template and extension at the 3' end of the DNA of interest by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

6) transcription of the double-stranded DNA sequence of (5) by the DNA-dependent RNA polymerase, to produce a RNA transcript complementary to the target DNA and having a defined 5' end;

7) hybridization of the reverse primer to the complementary 3' region of the RNA transcript of (6);

8) extension of the 3' end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (7) and a reverse primer extension product having a sequence which is homologous to the DNA of interest;

9) hydrolysis of the RNA transcript of (8) by the agent with RNase H activity;

10) hybridization of the 3' region of the promoter primer to a complementary 3' region of the reverse primer extension product of (8);

11) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3' end of

the reverse primer extension product by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a defined 5′ end, a defined 3′ end and a double-stranded promoter; and

12) transcription of the double-stranded DNA sequence having a defined 5′ end, a defined 3′ end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5′ end, a defined 3′ end and a sequence complementary to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

11. A method of Claim 10 wherein the agent with RNase H activity is ribonuclease H.

12. A method of Claim 10 wherein the restriction enzyme which recognizes double-stranded DNA and specifically cleaves outside its recognition site is Fok 1.

13. A method for amplifying DNA of interest having a defined 3′ end and contained within a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest

available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest;

c) a promoter primer complement oligonucleotide having a region complementary to the promoter sequence of the promoter primer;

d) a reverse primer having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

e) at least one RNA-dependent DNA polymerase;

f) at least one DNA-dependent DNA polymerase;

g) at least one DNA-dependent RNA polymerase;

h) an agent with RNase H activity; and

i) appropriate nucleoside triphosphates,

under conditions appropriate for:

1) hybridization of the promoter primer complement oligonucleotide to the promoter sequence of the promoter primer, to produce a double-stranded promoter complex;

2) hybridization of the double-stranded promoter complex to the defined 3' end of the DNA of interest;

3) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

4) transcription of the double-stranded DNA sequence of (3) by the DNA-dependent RNA polymerase, to produce a RNA transcript complementary to the target DNA and having a defined 5' end;

5) hybridization of the reverse primer to the complementary 3' region of the RNA transcript of (4);

6) extension of the 3' end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (5) and a reverse primer extension product having a sequence which is homologous to the DNA of interest;

7) hydrolysis of the RNA transcript of (6) by the agent with RNase H activity;

8) hybridization of the 3' region of the promoter primer to a complementary 3' region of the reverse primer extension product of (6);

9) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3' end of the reverse primer extension product by the DNA-dependent DNA polymerase using the promoter primer as a template, to produce a double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter; and

10) transcription of the double-stranded DNA sequence having a defined 5' end, a

defined 3'end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce an RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest, and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

14. A method of Claim 13 wherein the agent with RNase H activity is ribonuclease H.

15. A method for amplifying DNA of interest having a defined 3' end and contained in a target DNA sequence, comprising combining:

a) the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences;

b) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to the defined 3' end of the DNA of interest;

c) a reverse primer having a region complementary to a 3' region of a RNA transcript synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

d) a restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site;

e) a restriction oligonucleotide having a 3' region complementary to a region within the target DNA sequence adjacent to and partially overlapping the defined 3' end of the DNA of interest and a 5' region comprising a sequence which corresponds to the recognition site of the restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site;

f) at least one RNA-dependent DNA polymerase;

g) at least one DNA-dependent DNA polymerase;

h) at least one DNA-dependent RNA polymerase;

i) an agent with RNase H activity; and

j) appropriate nucleoside triphosphates,

under conditions appropriate for:

1) hybridization of the 3' region of the restriction oligonucleotide to the complementary region of the target DNA sequence;

2) cleavage of the target DNA sequence at a selected site by the restriction enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site, to produce DNA of interest having a defined 3' end;

3) hybridization of the 3' region of the promoter primer to the complementary 3' end of the DNA of interest;

4) extension of the 3' end of the promoter primer by the DNA-dependent DNA polymerase using the DNA of interest as a template and extension of the 3' end of the DNA of interest by the DNA-dependent DNA polymerase

using the promoter primer as a template, to produce a double-stranded DNA sequence having a double-stranded promoter;

5) transcription of the double-stranded DNA sequence of (4) by the DNA-dependent RNA polymerase, to produce a RNA transcript complementary to the target DNA and having a defined 5′ end;

6) hybridization of the reverse primer to the complementary 3′ region of the RNA transcript of (5);

7) extension of the 3′ end of the reverse primer by the RNA-dependent DNA polymerase using the RNA transcript as a template, to produce a heteroduplex molecule comprising the RNA transcript of (6) and a reverse primer extension product having a sequence which is homologous to the DNA of interest;

8) hydrolysis of the RNA transcript of (7) by the agent with RNase H activity;

9) hybridization of the 3′ region of the promoter primer to a complementary 3′ region of the reverse primer extension product of (7);

10) extension of the 3′ end of the promoter primer by the DNA-dependent DNA polymerase using the reverse primer extension product as a template and extension of the 3′ end of the reverse primer extension product by the DNA-dependent DNA polymerase, using the promoter primer as a template, to produce a double-stranded DNA sequence having a

defined 5' end, a defined 3' end and a double-stranded promoter; and

11) transcription of the double-stranded DNA sequence having a defined 5' end, a defined 3' end and a double-stranded promoter by the DNA-dependent RNA polymerase to produce a RNA transcript having a defined 5' end, a defined 3' end and a sequence complementary to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

16. A method of Claim 15 wherein the agent with RNase H activity is ribonuclease H.

17. A method of Claim 15 wherein the enzyme which recognizes single-stranded DNA and specifically cleaves outside its recognition site at a selected site is Mnl 1.

18. A method for amplifying DNA of interest having a defined 3' end and contained in a target DNA sequence, comprising:

a) combining the target DNA sequence, treated, if necessary, to render the DNA of interest available for hybridization with complementary nucleic acid sequences, with a DNA-dependent RNA polymerase which recognizes single-stranded DNA and synthesizes RNA using the DNA of interest as a template, such that a RNA/DNA heteroduplex molecule is produced and single-stranded RNA is

produced by the DNA-dependent RNA polymerase using the RNA/DNA heteroduplex as a template;

b) combining:

1) the RNA sequence synthesized using the DNA of interest as a template;

2) a promoter primer having a 5' region comprising a DNA-dependent RNA polymerase promoter sequence and a region 3' of the promoter sequence complementary to a 3' region of the RNA sequence synthesized using the DNA of interest as a template;

3) a reverse primer having a region complementary to the 3' end of the DNA of interest;

4) at least one RNA-dependent DNA polymerase;

5) at least one DNA-dependent DNA polymerase;

6) at least one DNA-dependent RNA polymerase;

7) an agent with RNase H activity; and

8) appropriate nucleoside triphosphates,

under conditions appropriate for:

i) hybridization of the promoter primer to the complementary 3' region of the RNA sequence synthesized by a DNA-dependent RNA polymerase using the DNA of interest as a template;

ii) extension of the 3' end of the promoter primer by the RNA-dependent DNA polymerase using the RNA sequence of (i) as a template, to produce a heteroduplex molecule comprising the

RNA sequence of (i) and a promoter primer extension product having a sequence which corresponds to the DNA of interest;

iii) hydrolysis of the RNA sequence of (ii) by the agent with RNase H activity;

iv) hybridization of the reverse primer to a complementary 3' region of the promoter primer extension product of (ii);

v) extension of the 3' end of the reverse primer by the DNA-dependent DNA polymerase using the promoter primer extension product as a template to produce a double-stranded DNA sequence having a defined 5' end and a double-stranded promoter; and

vi) transcription of the double-stranded DNA sequence having a defined 5' end and a double-stranded promoter by the DNA-dependent RNA polymerase, to produce a RNA transcript having a defined 5' end and a sequence which corresponds to the DNA of interest,

and maintaining the resulting combination under conditions appropriate for DNA of interest, thereby resulting in amplification of the DNA of interest.

19. A method of Claim 18 wherein the agent with RNase H activity is ribonuclease H.

20. A method of Claim 18 wherein the DNA-dependent RNA polymerase is selected from the group consisting of: T7 RNA polymerase, SP6 RNA polymerase and T3 RNA polymerase.

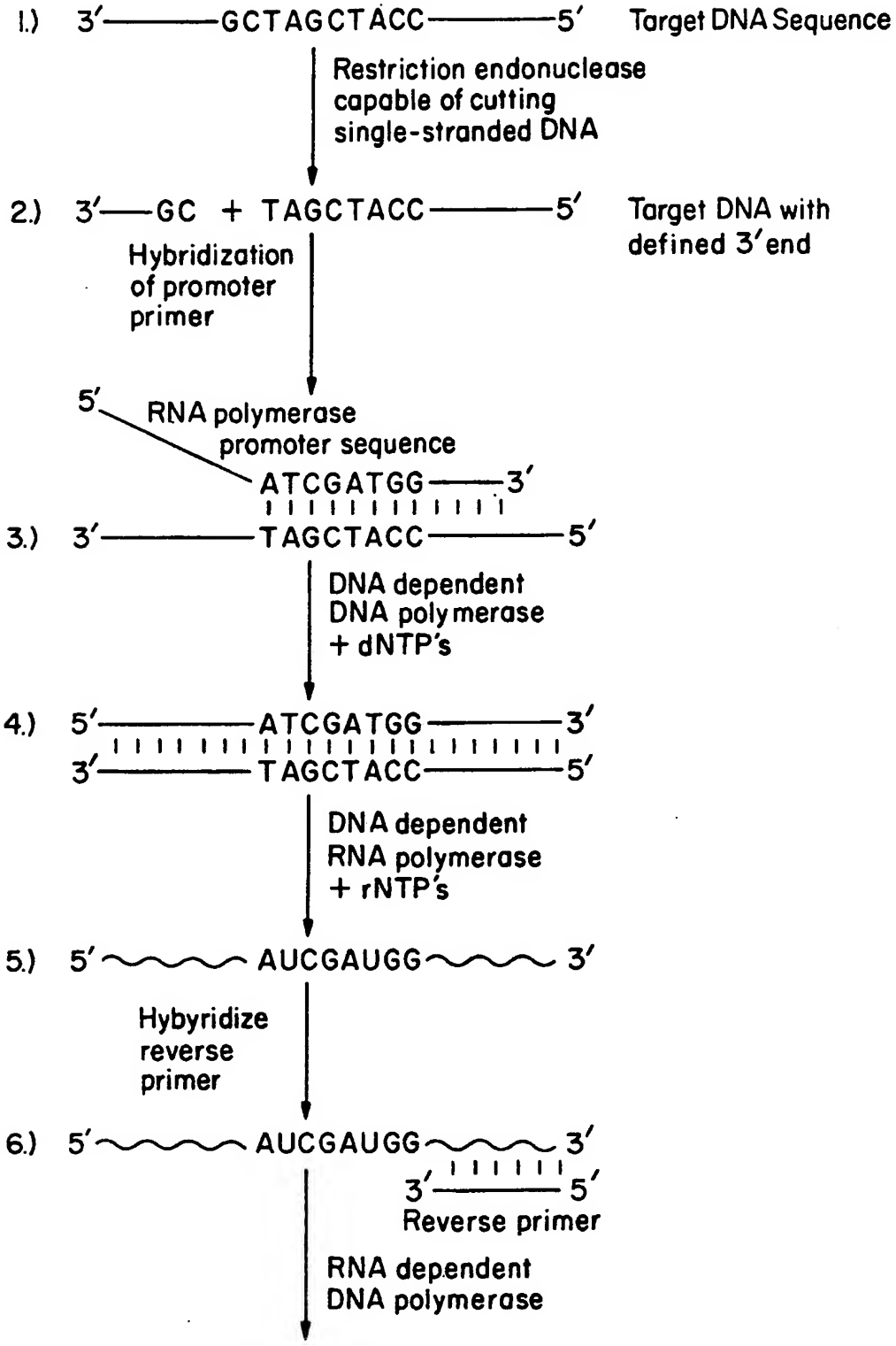

Fig. 1 SHEET 1 of 2

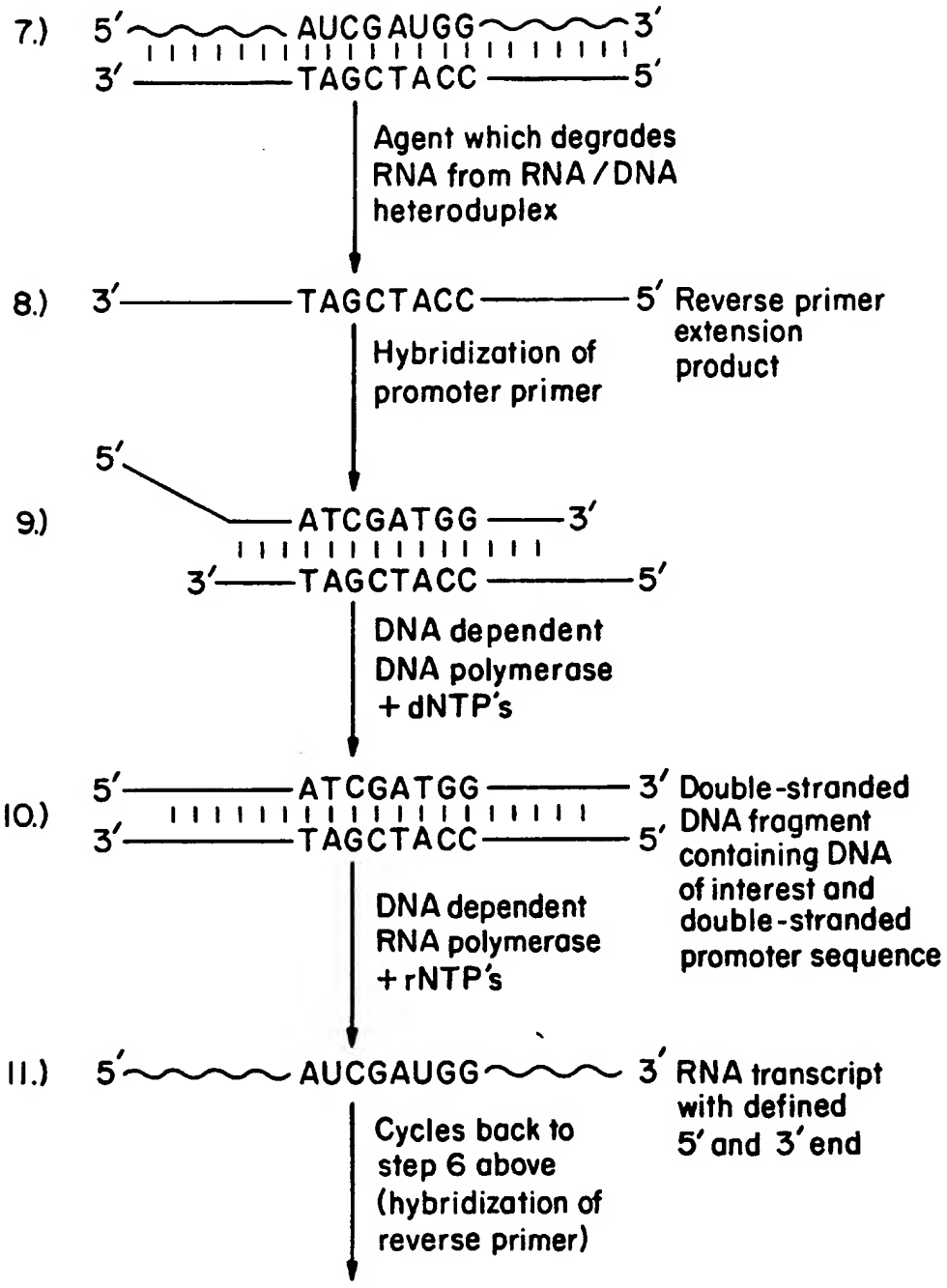

Fig. 1 SHEET 2 of 2

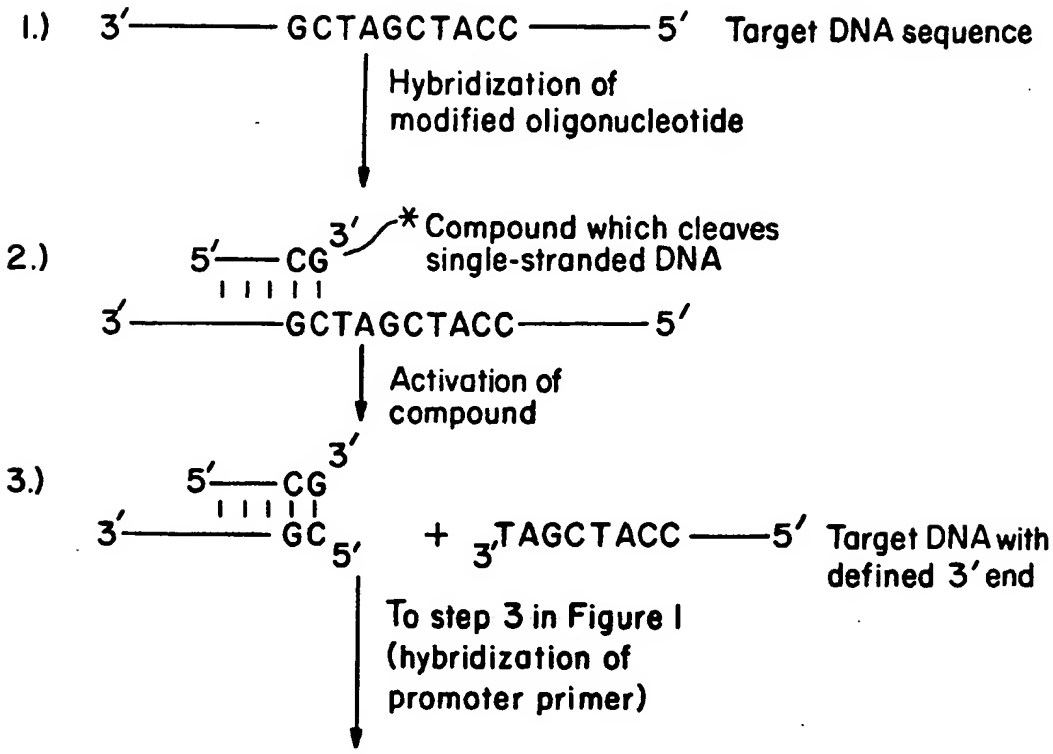
1.) 3′——— GCTAGCTACC ——— 5′ Target DNA sequence
Hybridization of modified oligonucleotide
2.) 5′— CG 3′ * Compound which cleaves single-stranded DNA
3′———GCTAGCTACC———5′
Activation of compound
3.) 5′— CG 3′
3′——— GC 5′ + 3′TAGCTACC ——5′ Target DNA with defined 3′ end
To step 3 in Figure I (hybridization of promoter primer)

Fig. 2

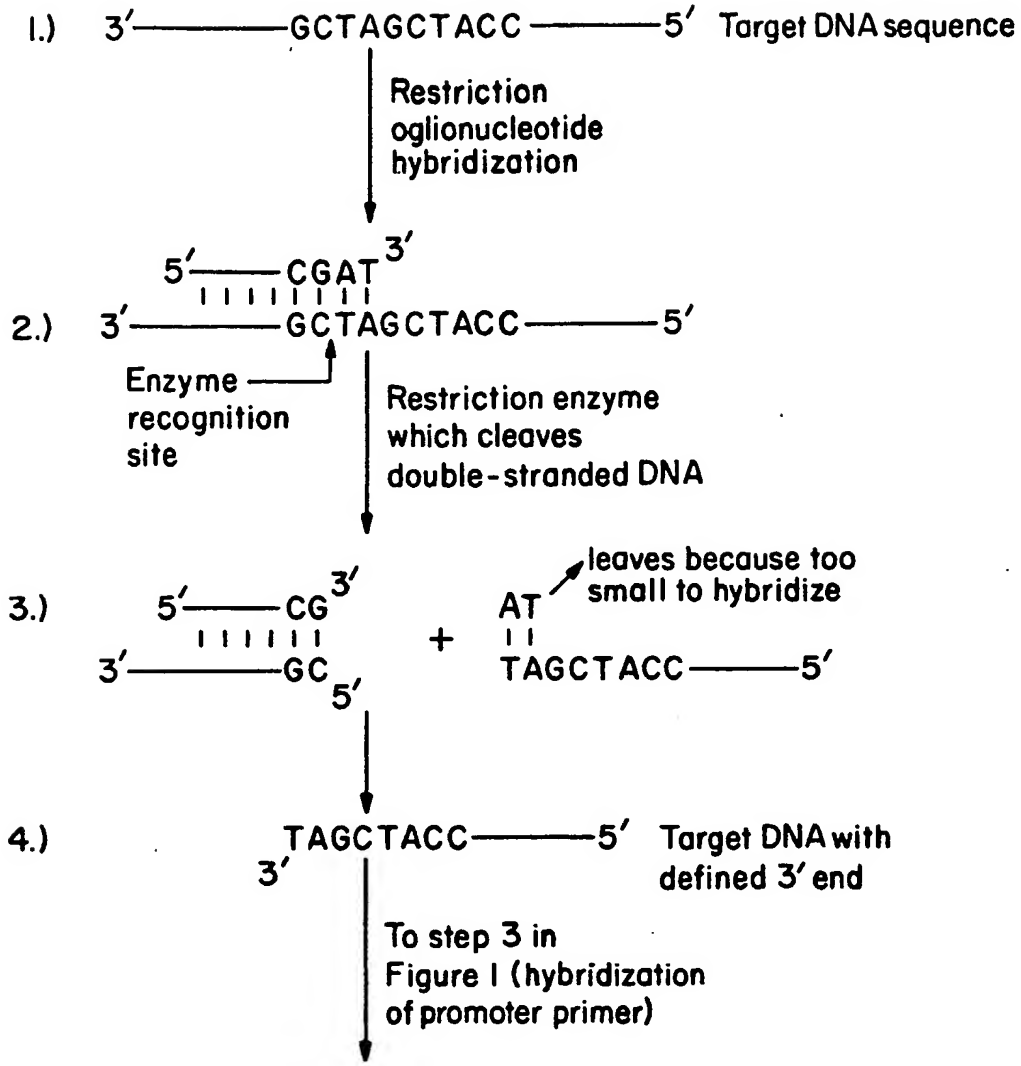
1.) 3′——GCTAGCTACC——5′ Target DNA sequence
Restriction oglionucleotide hybridization
5′——CGAT 3′
2.) 3′——GCTAGCTACC——5′
Enzyme recognition site
Restriction enzyme which cleaves double-stranded DNA
3.) 5′——CG 3′
3′——GC 5′
+
AT
leaves because too small to hybridize
TAGCTACC——5′
4.) TAGCTACC——5′ Target DNA with defined 3′ end
3′
To step 3 in Figure 1 (hybridization of promoter primer)

Fig. 3

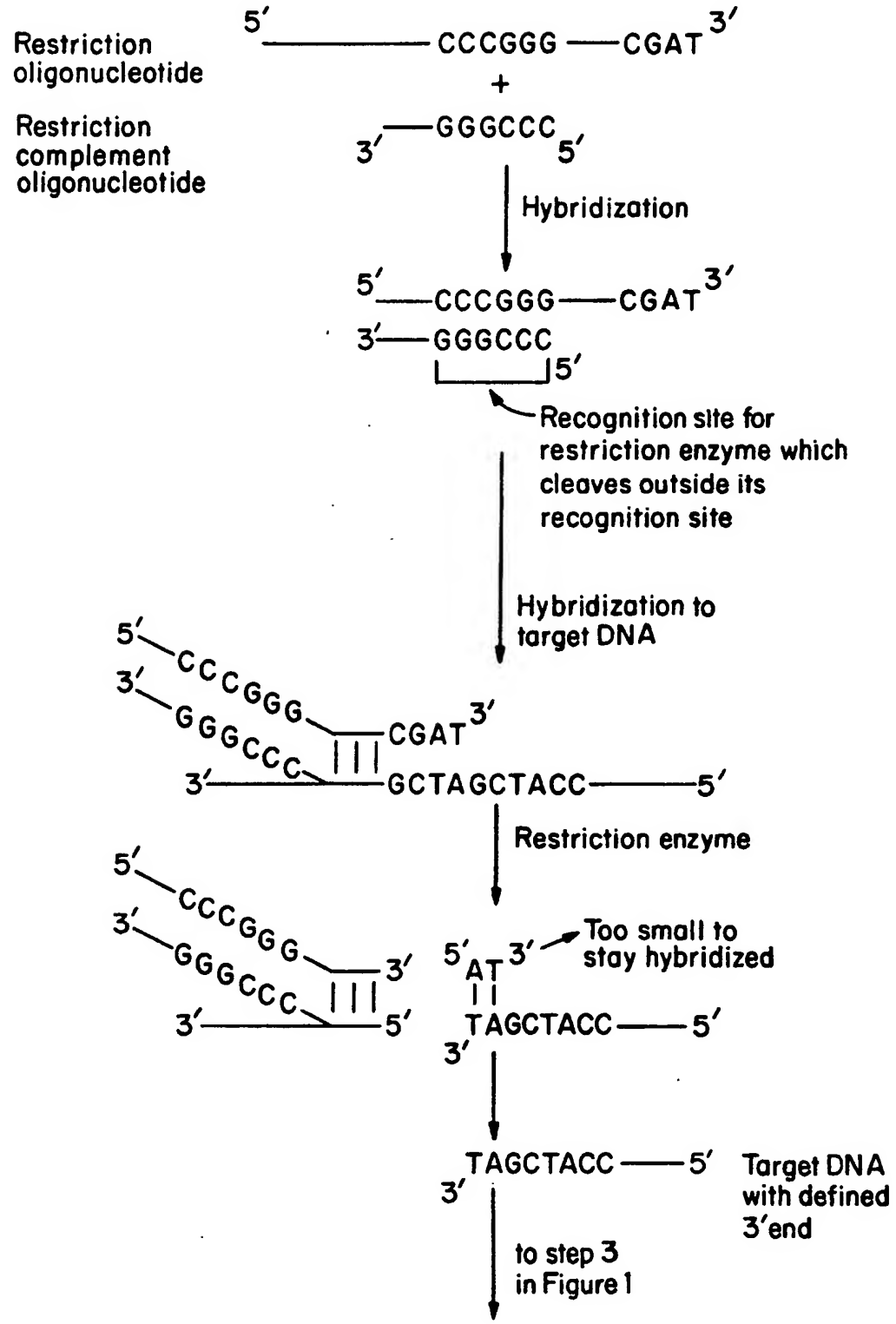
Restriction oligonucleotide
5′ CCCGGG CGAT 3′
+
Restriction complement oligonucleotide
3′ GGGCCC 5′
Hybridization
5′ CCCGGG CGAT 3′
3′ GGGCCC 5′
Recognition site for restriction enzyme which cleaves outside its recognition site
Hybridization to target DNA
5′ CCCGGG CGAT 3′
3′ GGGCCC GCTAGCTACC 5′
Restriction enzyme
5′ CCCGGG 3′
3′ GGGCCC 5′
5′ AT 3′
3′ TAGCTACC 5′
Too small to stay hybridized
TAGCTACC 5′
3′
Target DNA with defined 3′end
to step 3 in Figure 1

Fig. 4

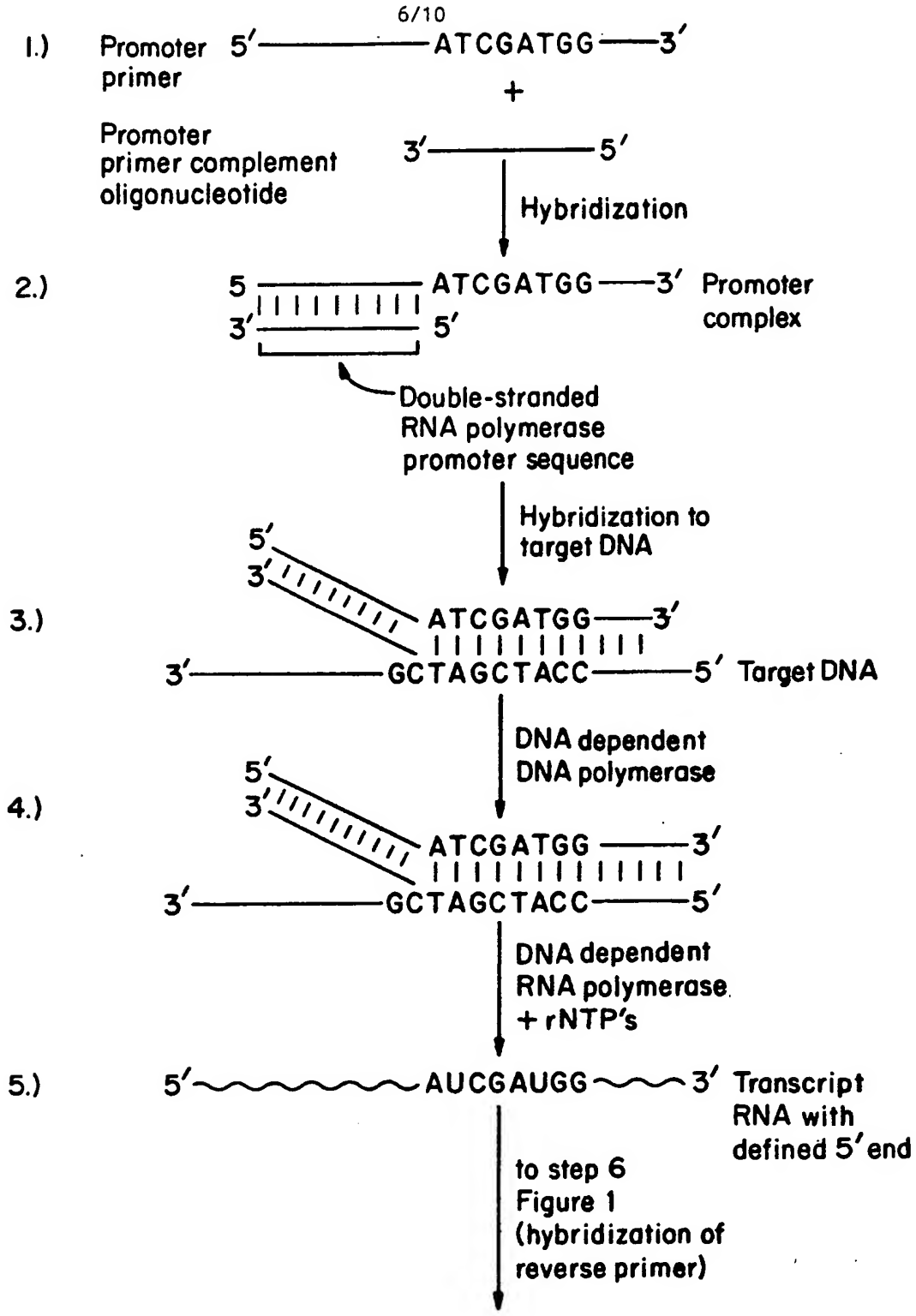
1.)
Promoter primer
5′ ATCGATGG 3′
+
Promoter primer complement oligonucleotide
3′ 5′
Hybridization
2.)
5 ATCGATGG 3′ Promoter complex
3′ 5′
Double-stranded RNA polymerase promoter sequence
Hybridization to target DNA
3.)
ATCGATGG
3′ GCTAGCTACC 5′ Target DNA
DNA dependent DNA polymerase
4.)
ATCGATGG
3′ GCTAGCTACC 5′
DNA dependent RNA polymerase + rNTP's
5.)
5′ AUCGAUGG 3′ Transcript RNA with defined 5′ end
to step 6 Figure 1 (hybridization of reverse primer)

Fig. 5

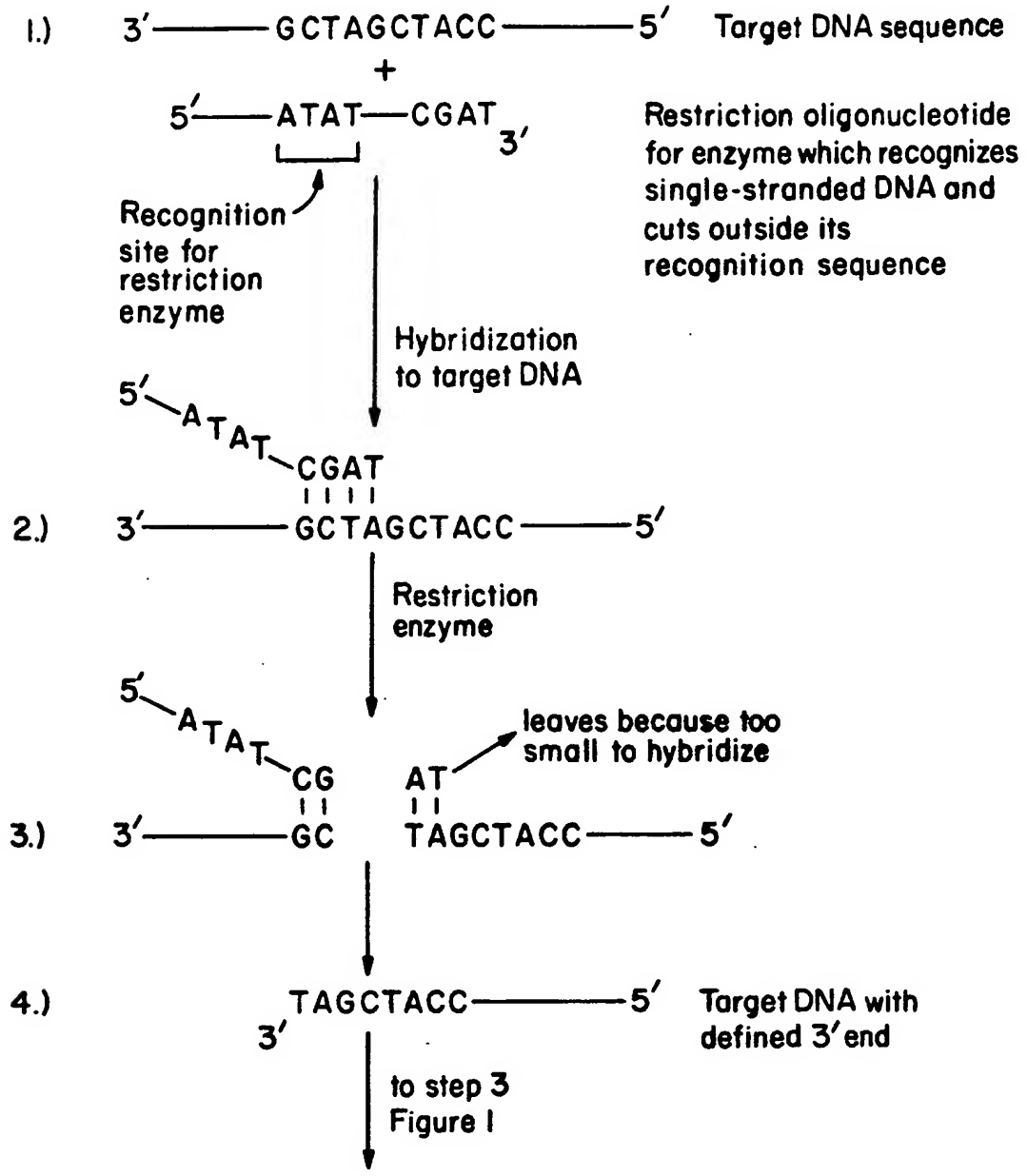
1.)
3′——GCTAGCTACC——5′
Target DNA sequence
+
5′——ATAT—CGAT 3′
Restriction oligonucleotide for enzyme which recognizes single-stranded DNA and cuts outside its recognition sequence
Recognition site for restriction enzyme
Hybridization to target DNA
2.)
3′——GCTAGCTACC——5′
Restriction enzyme
leaves because too small to hybridize
3.)
3′——GC
TAGCTACC——5′
4.)
TAGCTACC——5′
3′
Target DNA with defined 3′ end
to step 3 Figure I

Fig. 6

AMPLIFICATION of RNA

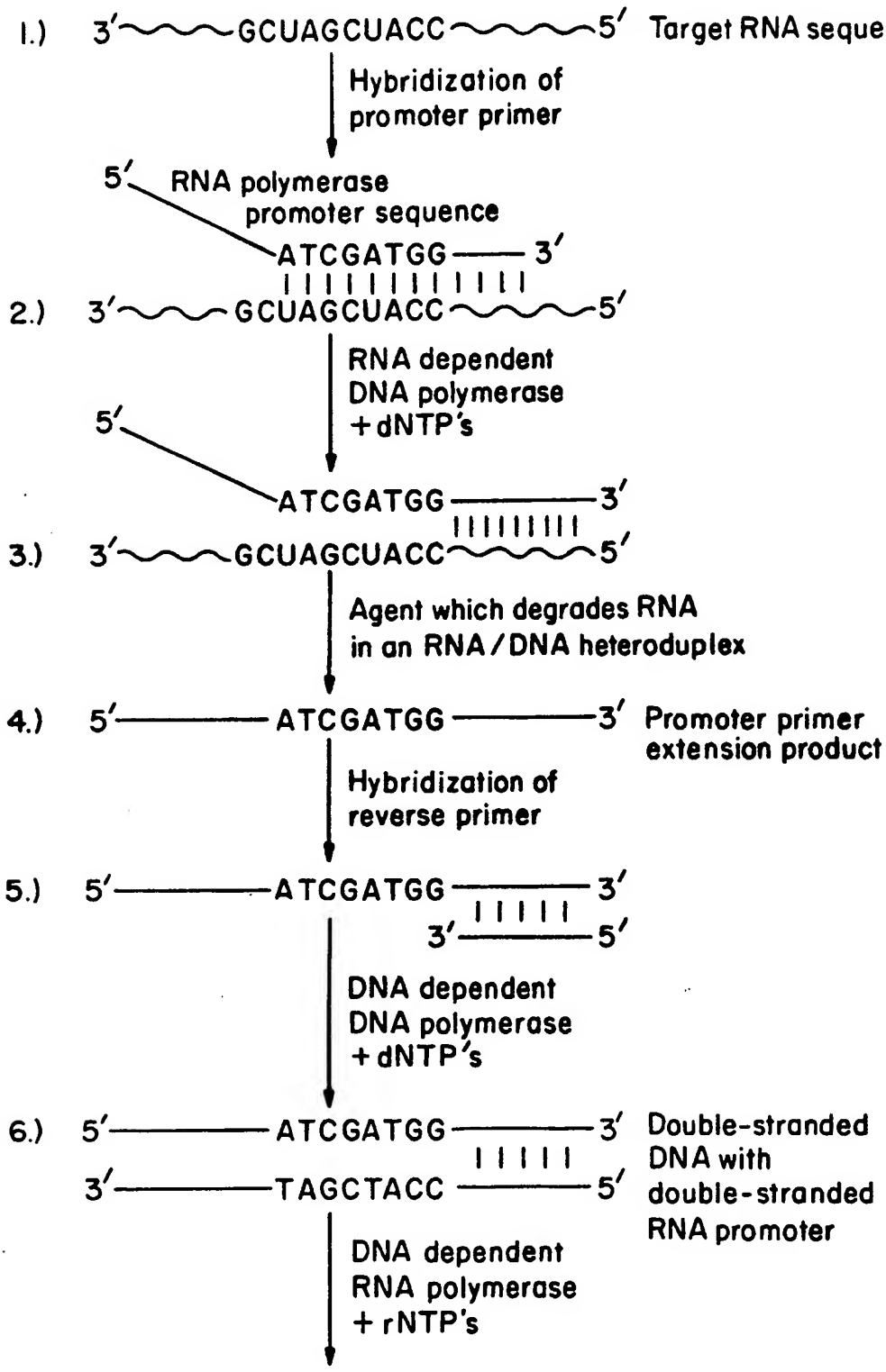

Fig. 7 SHEET 1 of 2

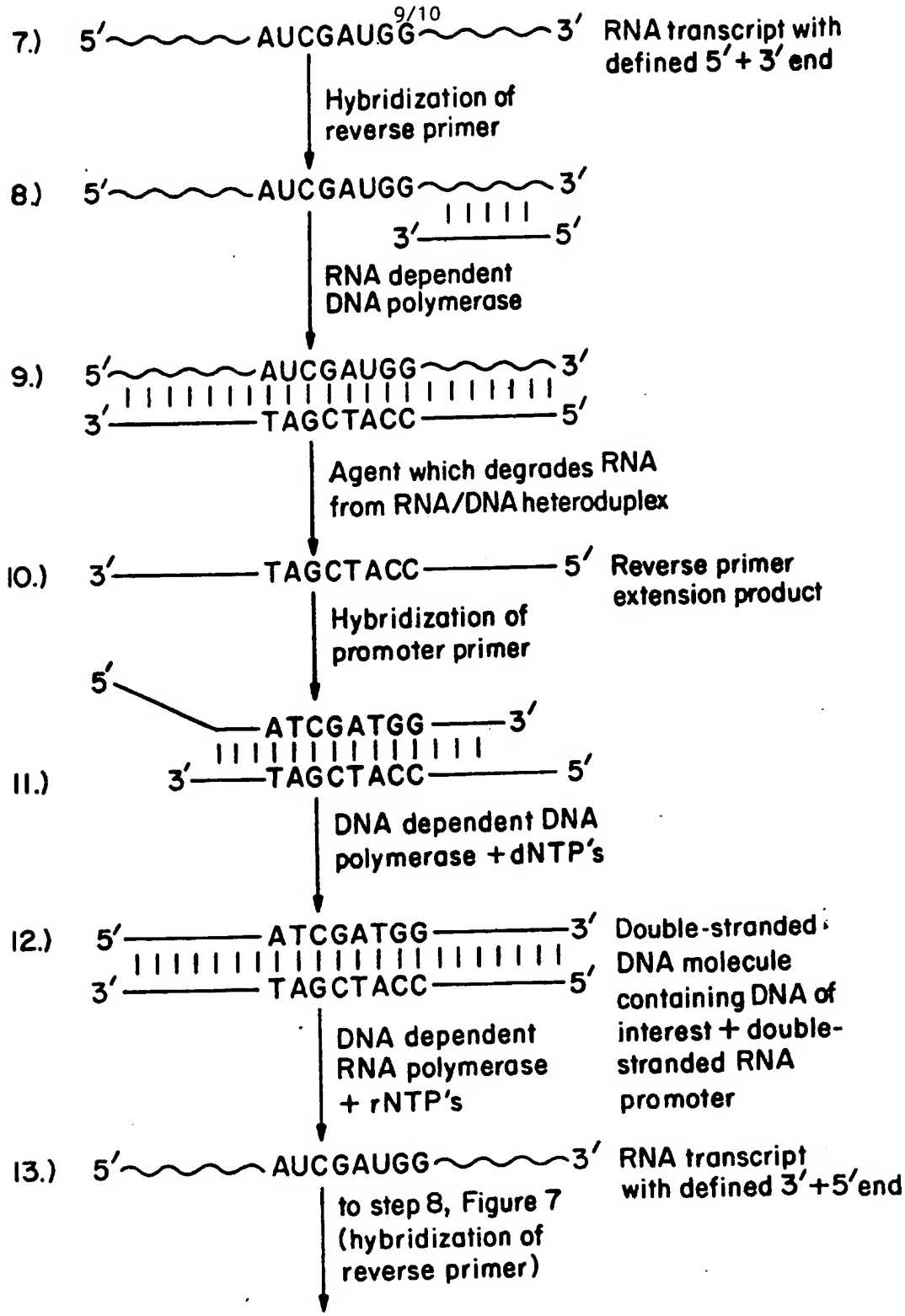

Fig. 7 SHEET 2 of 2

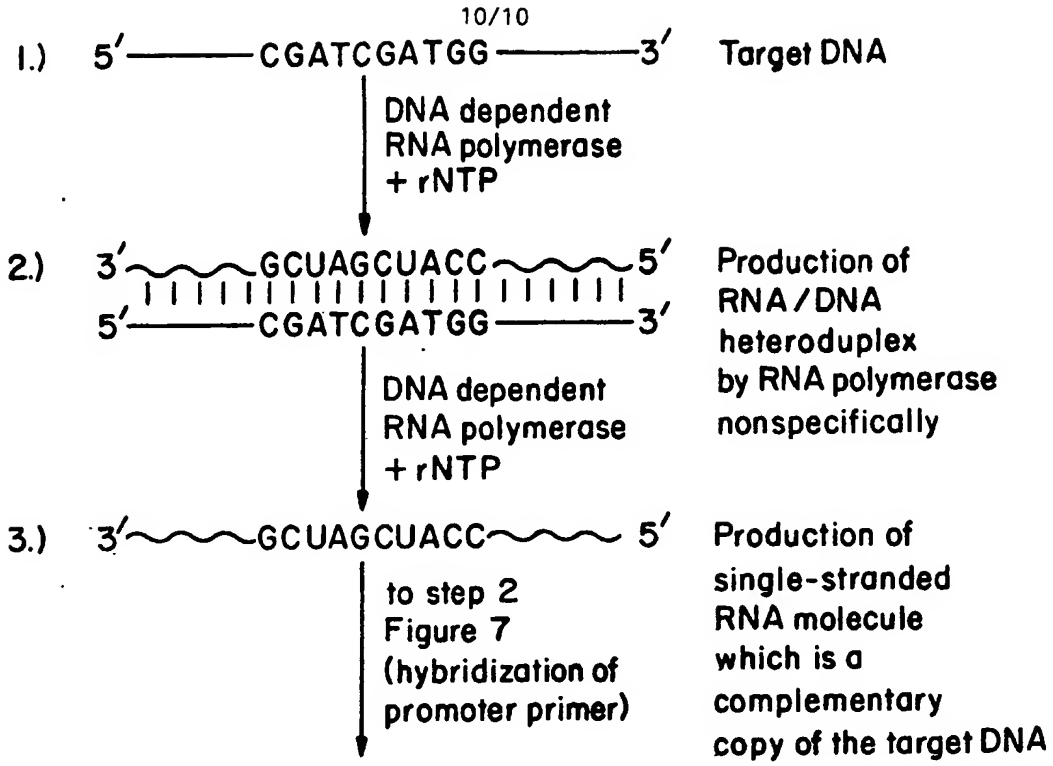
1.) 5′——CGATCGATGG——3′
Target DNA
DNA dependent
RNA polymerase
+ rNTP
2.) 3′~~GCUAGCUACC~~5′
5′——CGATCGATGG——3′
Production of
RNA/DNA
heteroduplex
by RNA polymerase
nonspecifically
DNA dependent
RNA polymerase
+ rNTP
3.) 3′~~GCUAGCUACC~~5′
Production of
single-stranded
RNA molecule
which is a
complementary
copy of the target DNA
to step 2
Figure 7
(hybridization of
promoter primer)

Fig. 8

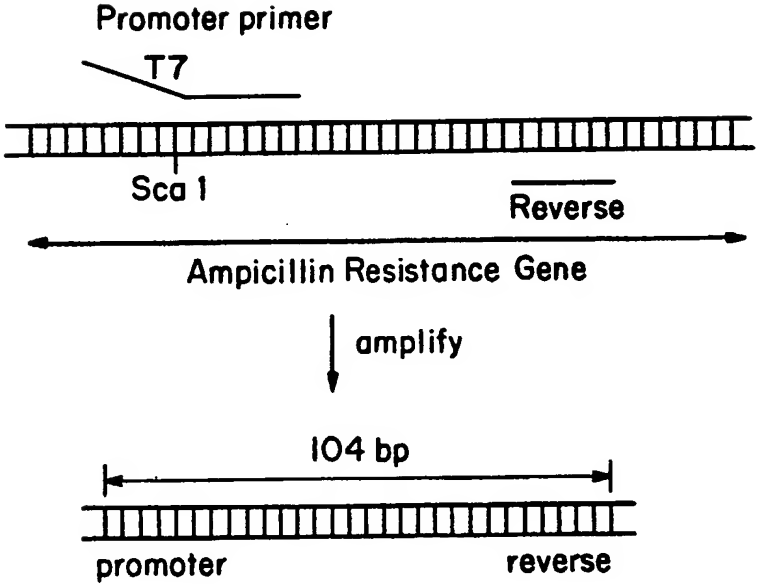
Promoter primer
T7
Sca 1
Reverse
Ampicillin Resistance Gene
amplify
104 bp
promoter
reverse

Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05321

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC[5]	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0329822 (CANGENE CORP.) 30 August 1989 see the whole document, especially page 3, line 15 - page 4, line 32; page 8, lines 10-46; claims	1-20
A	EP, A, 0310229 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 5 April 1989 see page 1, line 45 - page 7, line 30 (cited in the application)	1
A	Gene, volume 40, December 1985, Elsevier Science Publishers, W. Szybalski: "Universal restriction endonucleases: designing novel cleavage specificities by combining adapter oligodeoxynucleotide and enzyme moieties", pages 169-173 see abstract (cited in the application) ./.	10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th January 1991	06 FEB 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS J. JAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO, A, 90/06995 (SISKA DIAGNOSTICS, INC.) 28 June 1990 see page 6, line 35 - page 12, line 26; claims	1-20
	--	
P,X	Proc. Natl. Acad. Sci. USA, volume 87, March 1990, J.C. Guatelli et al.: "Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication", pages 1874-1878 see the whole article, especially the abstract; figure 1; page 1878, column 1, lines 1-29	1-20

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9005321 SA 40916

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/01/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0329822	30-08-89	JP-A- 2005864	10-01-90
EP-A- 0310229	05-04-89	AU-A- 2318188	01-03-89
		JP-T- 2501532	31-05-90
		WO-A- 8901050	09-02-89
WO-A- 9006995	28-06-90	AU-A- 4829690	10-07-90
		EP-A- 0373960	20-06-90